University of Rostock

Aus der
Klinik und Poliklinik für Herzchirurgie am Universitätsklinikum Rostock
Direktor: Prof. Dr. med. habil. Gustav Steinhoff

Stammzell -Therapie nach Myokardinfarkt: direkte Applikation oder Medikamenten-vermittelte Rekrutierung?

Dissertation

zur Erlangung des akademischen Grades

Doctor rerum naturalium (Dr. rer. nat.)

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität Rostock

Vorgelegt von

Dario Furlani

geboren am 28.08.1976 in Monza, Italien

Rostock, September 2009

urn:nbn:de:gbv:28-diss2010-0045-6

From the
Clinic and Policlinic for Cardiac Surgery at the University of Rostock
Director: Prof. Dr. med. habil. Gustav Steinhoff

Stem Cells therapy after myocardial infarction: direct application or drug-mediated recruitment?

Dissertation

to obtain the academic degree

Doctor rerum naturalium (Dr. rer. nat.)

at the Faculty of Mathematics, Physics and Natural Sciences

of University of Rostock

Submitted by

Dario Furlani

born on 28.08.1976 in Monza, Italy

Rostock, September 2009

urn:nbn:de:gbv:28-diss2010-0045-6

Die vorliegende Arbeit entstand in der Zeit von April 2006 bis August 2009 an FKGO Forschungslaboratorien für kardialen Gewebe- und Oganersatz der Klinik und Poliklinik für Herzchirurgie am Universitätsklinikum Rostock.

Dekan: Prof. Dr. Hendrik Schubert

1. Gutachter: Prof. Dr. med. habil. Gustav Steinhoff

2. Gutachter: Prof. Dr. Dieter Weiss

3. Gutachter: Prof. Dr. med. Dr. rer. nat. Wolfgang Wagner

“se il cuore fosse solamente sangue e citochine, non sarei mai arrivato qui

a Carolin”

Contents

Contents……v
List of publications included in the dissertation……vi
List of abbreviations……vii
Zusammenfassung……ix
Summary……xi
1. **Introduction**……1
2. **Acute myocardial infarction**……3
   2.1. Scenario……3
   2.2. Inflammative response……6
   2.3. Ischemia/reperfusion injury……10
   2.4. Cardiac remodeling……13
   2.5. Therapies for acute myocardial infarction……17
   2.6. What to target?……20
3. **Stem cell mediated cardiac repair**……21
   3.1. Cardiac regeneration……21
   3.2. Stem cell therapies and stem cell sources……23
   3.3. Haematopoietic stem cell mediated cardiac repair……28
   3.4. Mesenchymal stem cell and myocardial regeneration……29
   3.5. Mechanisms of stem cell mediated cardiac repair……30
   3.6. Challenges and future directions……30
4. **Drugs driven stem cell mobilization**……33
   4.1. Granulocyte colony-stimulating factor……33
   4.2. Erythropoietin……34
5. **Molecular mechanisms of stem cell mobilization and homing**……40
   5.1. SDF-1α mediated mechanisms of stem cell mobilization……40
   5.2. SDF-1α and EPO mediated mechanisms of SCs transmigration……42
   5.3. eNOS and EPO mediated mechanisms of SCs mobilization and homing……43
   5.4. SDF-1α and CXCR4 axis in the heart……45
6. **Stem cell administration route**……48
   6.1. Intraarterial application……48
   6.2. Intravenous infusion……51
   6.3. Direct injection in the ventricular wall……51
7. **Safety of stem cell treatments**……52
   7.1. How to control a highly potent cell?……52
   7.2. Mesenchymal stem cell and cancer……52
   7.3. Mesenchymal stem cell quality control……55
8. **Conclusion**……57
9. **References**……59
10. **Financial support**……79
11. **Publications list**……80
12. **Abstract presentations and conferences**……83
13. **Acknowledgement**……85
14. **Manuscripts reprints**……87

List of publications included in the dissertation

Manuscript 1

Klopsch C*, **Furlani D***, Gäbel R, Wagner K, Li W, Ugurlucan M, Kundt G, Zingler C, Titze U, Wang W, Ong LL, Li R, Ma N, Steinhoff G. Intracardiac Injection of Erythropoietin Induces Stem Cell Recruitment and Improves Cardiac Functions in a Rat Myocardial Infarction Model. *J Cell Mol Med*. 2009;13(4):664-79

*Equal contribution

Impact Factor: 6.807

Manuscript 2

Furlani D, Klopsch C, Gäbel R, Ugurlucan M, Pittermann E, Klee D, Wagner K, Li W, Wang W, Ong LL, Nizze H, Titze U, Lutzow K, Lendlein A, Steinhoff G, Ma N. Intracardiac erythropoietin injection reveals antiinflammatory potential and improved cardiac functions detected by Forced Swim Test. *Transplant Proc*. 2008;40:962-6

Impact Factor: 1.027

Manuscript 3

Furlani D, Ugurlucan M, Ong LL, Bieback K, Pittermann E, Westien I, Wang W, Yerebakan C, Li W, Gäbel R, Li R-K, Vollmar B, Steinhoff G, Ma N. Is the intravascular administration of mesenchymal stem cells safe? *Microvascular Research.* 2009;77(3):370-6. Epub 2009 Feb 26

Impact Factor: 2.365

Manuscript 4

Furlani D, Li W, Pittermann E, Klopsch C, Wang L, Knopp A, Jungebluth P, Thedinga E, Havenstein C, Westien I, Ugurlucan M, Li R, Ma N, Steinhoff G. A transformed cell population derived from cultured mesenchymal stem cells has no functional effect after transplantation into the injured heart. *Cell Transplantation*. 2009;18(3):319-31.

Impact Factor: 5.251

List of abbreviations

CHF	Congestive heart failure
BM	Bone marrow
MSCs	Mesenchymal stem cells
EPO	Erythropoietin
SDF-1α	Stromal-derived factor-1 alpha
CXCR4	CXC chemokine receptor 4
MI	Myocardial infarction
PCI	Percutaneous coronary intervention
LV	Left ventricle
LDL	Low density lipoprotein
VCAM-1	Vascular cell adhesion molecule-1
MCP-1	Monocyte chemotactic protein-1
TNF-α	Tumor necrosis factor alpha
IL	Interleukin
MAPK	Mitogen-activated protein kinases
JAK	Janus kinase
STAT	Signal transducers and activator of transcription
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
AP-1	Activator protein 1
C/AATT	C/AATT-enhancer binding protein
gp130	Glycoprotein 130
PPAR-γ	Peroxisome proliferator-activated receptors
TLR	Toll-like receptor
CD	Cluster of differentiation
ROS	Reactive oxygen species
H_2O_2	Hydrogen peroxide
HMGB1	High-mobility group box 1,
RAGE	Receptor for advanced glycation endproducts
LPS	Lipopolysaccharides
Akt	Akt/protein kinase B
SCF	Stem cell factor
Ca^{2+}	Calcium
ATP	Adenosine-5'-triphosphate
Na^{+}	Sodium cation
K^{+}	Potassium cation
H^{+}	Hydron
HCO^{3-}	Hydrogencarbonate anion
H_2O	Water
NO	Nitric oxide
DNA	Deoxyribonucleic acid
TGF	Transforming growth factor
MMPs	Matrix metalloproteinases
AT1	Angiotensin receptor 1
OPN	Osteopontin
VEGF	Vascular endothelial growth factor
bFGF	Basic fibroblast growth factor
Ang-2	Angiopoietin 2
HGF	Hepatocyte growth factor
iNOS	Inducible nitric oxide synthase
ELR	Glutamic acid-leucine-arginine motif
IP-10	Interferon-gamma-induced protein 10
CABG	Coronary artery bypass graft surgery
CSCs	Cardiac stem cells
BMC	Bone-marrow-derived cell

BMMNC	Bone-marrow mononuclear cell
CPC	Circulating progenitor cell
EPC	Endothelial progenitor cell
SMB	Skeletal myoblast
TEIM	Transendocardial intramyocardial
VEGFR2	Kinase insert domain receptor (KDR)
ES	Embryonic stem
Kit	C-kit receptor (CD117)
Sca 1	Ataxin 1
Isl 1	Islet-1 transcription factor
Oct 3/4	Octamer 3/4
Klf 4	Krüppel-like family of transcription factor 4
Sox 2	Sex determining region Y-box 2
cMyc	Myelocytomatosis oncogene
iPS	Induced pluripotent stem
MN	Mononuclear
BMMSCs	Bone-marrow-derived mesenchymal stem cells
G-CSF	Granulocyte colony-stimulating factor
Bcl 2	B-cell lymphoma 2
mRNA	Messenger ribonucleic acid
cTnT	Cardiac troponin t
RV	Right ventricle
eNOS	Endothelial nitric oxide synthase
HSCs	Haematopoietic stem cells
SEM	Standard error mean
MIC	Myocardial infarction control
Pmax	Maximum pressure
dp/dt max	Peak rate of maximum pressure rise
-dp/dt max	Peak rate of maximum pressure decline
EDV	Enddiastolic volume
ESV	Endsystolic volume
SV	Stroke volume
EF	Ejection fraction
SW	Stroke work
HR	Heart rate
NIZ	Non-infarcted zone
IZ	Infarcted zone
DAPI	4',6-diamidino-2-phenylindole
SCs	Stem cells
VLA	Very late antigen
SD	Standard deviation
ICAM-1	Intercellular adhesion molecule-1
LFA	Lymphocyte function-associated antigen
NOS	Nitric oxide synthases
L-NAME	N-nitro-L-arginine-methylester-hydrochloride
TO-PRO3	TO-PRO 3 Iodide (family of monomeric, cyanine nucleic acid stains)
SVEC	Simian virus 40-transformed mouse endothelial cell line
sKitL	Soluble Kit ligand
mKitL	Membrane-bound kit ligand
PI3K	Phosphoinositide 3-kinases
EPOR	Erythropoietin receptor
SCID	Severe combined immunodeficiency
CFDA SE	Carboxyfluorescein diacetate succinimidyl ester
LAD	left anterior descending artery
GMP	Good Manufacturing Practice
BrdU	Bromodeoxyuridine (5-bromo-2-deoxyuridine)
PI	Propidium iodide

Zusammenfassung

Die chronische Herzinsuffizienz ist eine der häufigsten Ursachen für Morbidität und Mortalität in den Industrieländern. Sie entsteht als eine späte Folge verschiedener kardiovaskulärer Erkrankungen und ist durch den Verlust kontraktilen Muskels, durch Volumen- und Druck-Überlastung oder deren Kombination charakterisiert. Die Hauptmechanismen, welche zum Verlust kontraktilen Gewebes führen, sind Ischämie und oxidativer Stress. In Abhängigkeit von Dauer und Schweregrad können vereinzelt und periodisch auftretende Ereignisse myokardialer Ischämie und Reperfusion zu Zellschädigung und Zelltod führen. Die begrenzte mitotische Kapazität schränkt die autogene Regeneration des ischämischen Myokards deutlich ein, vielmehr kommt es in diesen Gebieten zu einem inadäquaten Ersatz durch fibrotisches Gewebe. Fortschritte in der Stammzellbiologie und klinische Versuche zeigen, dass kardiales Gewebe durch intrakardiale Gabe von Knochenmarksstammzellen oder durch gerichtete Mobilisierung vordifferenzierter Progenitorzellen aus dem Knochenmark regeneriert werden kann.

Stammzellen könnten den Heilungsprozess im beschädigten Herzen durch Erhöhung der Neoangiogenese und/oder durch Regeneration kardialer Myozyten im beschädigten Herzen verstärken. Erste klinische Ansätze, welche die Transplantation von Stammzellen mit einbeziehen, werden derzeit bei einer noch limitierten Anzahl von Patienten mit chronischer Herzinsuffizienz getestet. Der mögliche Transfer von Behandlungen mit Stammzellen in die klinische Praxis, erfordert die Möglichkeit zur Behandlung mit standardisierten, gut charakterisierten Zellen definierten Ursprungs ohne tumorogenes Potential. Der alleinige Einsatz dieser Zellen oder der Einsatz in Kombination mit Wirkstoffen, welche die Rekrutierung endogener Stammzellen stimulieren, könnten zukünftig helfen, kardiovaskuläre Erkrankungen zu behandeln oder deren Entstehung möglicherweise sogar zu verhindern.

Derzeit werden sowohl zelluläre als auch pharmakologische Strategien zur Anreicherung von Stammzellen im erkrankten Herzgewebe entwickelt. Die zugrundeliegenden Mechanismen der Medikamenten-vermittelten Stammzell-Rekrutierung im Herzgewebe durch Erythropoietin (EPO) wurden in dieser Arbeit *in vitro* und *in vivo* untersucht. Weiterhin wurden Transplantatationstechniken und Nebenwirkungen von kultivierten mesenchymalen Stammzellen *in vitro* und *vivo* evaluiert.

Die Ziele der Untersuchungen waren:

1. Die Evaluierung der therapeutischen Wirksamkeit intrakardialer Erythropoietin Injektion auf das infarzierte Herz.
2. Die Beurteilung, ob eine intrakardiale Injektion von EPO die Rekrutierung Stamm- und Progenitorzellen im infarzierten Herz durch Aktivierung von Signalketten beeinflussen

und so zusätzlich zur kardialen Regeneration nach myokardialem Infarkt (MI) beitragen kann.

3. Die Untersuchung des unmittelbaren Verhaltens mesenchymaler Stammzellen aus dem Fettgewebe nach intraarterieller Gabe und deren Eignung zur intravaskulären Transplantation.
4. Die Evaluierung des Effekts der spontanen Transformation mesenchymaler Stammzellen (MSCs) auf die kardiale Funktion.

Die wichtigsten Ergebnisse waren folgende:

1. Eine intrakardiale EPO Injektion stellt die myokardiale Funktionen wieder her, reduziert Apoptose, induziert Angiogenese und vermindert Fibroseprozesse nach MI.
2. EPO vermittelt positive Effekte durch die Rekrutierung von c-Kit$^+$ und CD34$^+$ Stammzellen.
3. Die Mobilisierungs- und Rekrutierungsaktivität von Knochenmarkstammzellen durch EPO wird durch die Interaktion der SDF-1α/CXCR4 Verbindung, eNOS und MMP-2 vermittelt.
4. Die unmittelbare Stimulierung durch EPO induziert die Hochregulierung zentraler Adhäsionsmoleküle in endothelialen Zellen.
5. EPO moduliert die SDF-1α/CXCR4 Verbindung in endothelialen Zellen und Kardiomyoblasten *in vitro.*
6. Die kardioprotektive Wirkung von EPO steht in engem Zusammenhang mit der Hochregulierung nachgeordneter Faktoren wie eNOS und Akt.
7. Abhängig von der Zellgröße kann eine intraarterielle Gabe von MSCs zu Verschlüssen distaler Blutgefäße führen. Eine pulmonale Sequestrierung kann bei kleinen Labortieren zum Tod führen.
8. Nach regulären Protokollen isolierte und unter Standardbedingungen kultivierte MSCs transformieren in frühen Passagen. Die transformierten Zellen zeigen keine therapeutische Wirkung nach der Transplantation im experimentellen Modell des myokardialen Infarkts.

In der vorliegenden Arbeit wurden neue Informationen im Feld der kardialen Stammzelltherapie dargelegt. Es wird gezeigt, dass die Behandlung mit EPO bei kardialer Ischämie die frühe kardiale Protektion und Stammzellrekrutierung vermitteln kann. Die dargestellten Ergebnisse können die Entwicklung von neuen Therapiestrategien des akuten Myokardinfarktes beeinflussen. Die aufgezeigten möglichen Nebenwirkungen der Zellkulturexpansion von Stammzellen und deren Zellapplikationstechniken erfordern *in-vitro* und *-vivo* Überprüfung vor einem klinischen Einsatz.

Summary

Congestive heart failure (CHF) is one of the most significant causes of morbidity and mortality in developed countries. It occurs as a late manifestation in diverse cardiovascular diseases characterized by the loss of contractile muscle and/or by volume and pressure overload. The major mechanisms involved in contractile tissue injury and loss are ischemia and oxidative stress. Depending on the duration and severity, single or intermittent episodes of myocardial ischemia and reperfusion may cause cell damage and death. The limited mitotic capacity of cardiomyocytes restricts the autogenous repair of the ischemic myocardium leading to replacement by fibrotic tissue. New advances in stem cell biology and clinical evidence demonstrated that the damaged cardiac tissue could be repaired either by intra-cardiac administration of bone marrow stem cells or by directional mobilization of lineage-committed stem cell from bone marrow (BM).

Stem cells could ameliorate this injury process by replenishing vascular supply (neoangiogenesis) and/or regenerating cardiac myocytes in the damaged heart. However, clinical approaches which employ stem cell transplantation procedures are currently being tested in limited sets of patients with ischemic heart disease. The eventual translation of stem cell technologies into clinical practice, however, will likely be based on treatments with the standardized, well characterized cell sources without undesirable tumorigenesis and/or drugs which stimulate the endogenous stem cell recruitment for the treatment and the possible prevention of cardiovascular disease.

Currently, both cellular and pharmacological strategies are developed to enhance stem cells in diseased cardiac tissue. The underlying mechanism of drug-mediated stem cell recruitment to heart tissue by erythropoietin (EPO) was investigated in this study *in vitro* and *in vivo*. Furthermore, transplantation techniques and side-effects of cultured mesenchymal stem cells were evaluated *in vitro* and *in vivo*.

The aims were:

1. To evaluate the therapeutic efficacy of intracardiac erythropoietin injection in the infarcted heart.
2. To assess if intracardiac injection of EPO could recruit stem and progenitor cells to the infarcted heart by activating stem cell homing signalling which promotes cardiac regeneration after myocardial infarction.
3. To investigate the immediate term behaviours of human adipose derived mesenchymal stem cells after intraarterial administration and their suitability for intravascular transplantation.

4. To evaluate effects of mesenchymal stem cells (MSCs) spontaneous transformation on the cardiac function.

The main findings are the following:

1. Intracardiac EPO injection restores myocardial functions, reduces apoptosis, induces angiogenesis and decreases fibrosis following MI.
2. EPO exerts its beneficial action directly and through early recruitment of c-Kit^{+} and CD34^{+} stem cells.
3. The mobilization and homing activity of EPO is mediated by the action of the SDF-1α/CXCR4 axis, eNOS and MMP-2.
4. EPO direct stimulation induces upregulation of central adhesion molecules in endothelial cells.
5. EPO modulates the SDF-1α/CXCR4 axis in endothelial cell and cardiomyoblast *in vitro.*
6. EPO cardioprotective capacity is closely related with the down stream up-regulation of cytoprotective factors such as eNOS and Akt.
7. Intraarterial MSCs administration may lead to occlusion in the distal vasculature due to their large size. Pulmonary sequestration may cause death in small laboratory animals.
8. MSCs isolated according usual protocols and cultured under standard conditions could undergo transformation in early passage culture. The transformed cells lose their therapeutic capacity after transplantation in the artificial myocardial infarction model.

In the present thesis, we provided novel information to the field of stem cell cardiac therapy. We suggested EPO treatment during coronary interventions or cardiac surgery might promote early cardiac protection and stem cell recruitment. Our findings have marked translational implications for new therapeutical strategies in acute myocardial infarction. The demonstrated potential side-effects of cell culture expansion of stem cells and their cellular application techniques require *in vitro* and *in vivo* testing before clinical administration.

1. Introduction

Recently, remarkable progresses have been made in cardiovascular field and in regenerative medicine [1] [2] [3] [4] [5]. However, myocardial infarction, peripheral arterial disease and angina pectoris have been continually on the rise among a background of increases in hypertension, hyperlipidaemia, diabetes and hyperuricaemia [6]. Moreover, at the dawn of the third millennium congestive heart failure following cardiovascular diseases is still the predominant cause of decease in developed countries. The adverse remodelling scenario that leads to heart failure is the result of contractile muscle damage and loss due to episodes of ischemia and oxidative stress. The ruinous consequences of this direct or indirect cell loss (cardiac necrosis), beside contractile failure, ventricular remodelling and arrhythmia, originate the development of cardiac insufficiency, disability and death. The extension of cardiac myocytes necrosis is caused by acute coronary occlusion and depends not only on the dimension of affected area but also on ischemia severity and duration.

Strategies to restore the injured heart and further regenerate functional tissue have been the major aims of physicians for decades. In the last years, a significant medical advance has been produced and the survival and life quality of patients with coronary disease have been considerably improved. In patients with acute myocardial infarction (MI), the conventional treatments consist in the redirection of blood flow in order to attempt restoration process (reperfusion therapy). The time interval before the restitution of the blood flow (ischemia duration) is the main determinant of the reperfusion therapy outcome. Such knowhow brought the physicians to invest bigger efforts in decreasing the time passing between symptoms manifestation in patients and therapeutic reperfusion application. However, it is progressively harder to achieve an additional reduction of this interval. Even if several advancements have been done in recanalization of obstructed coronary arteries, the majority of patients with acute coronary disease continue to display different extents of myocardial necrosis.

In addition, reperfusion therapy either by thrombolytic agents or invasive practices is not a guaranty of ischemic cell survival. A number of studies carried on during the last two decades, undoubtedly demonstrated the paradox of revascularization. Indeed, though reperfusion is the only possible option to preserve the ischemic tissue from certain death, a consistent part of cell necrosis is caused by blood flow restoration. This phenomenon known as reperfusion damage has been extensively investigated in

different experimental models but its relevance in the clinical context has been recognized lately.

The possibility to ameliorate efficacy of thrombolysis and percutaneous coronaries interventions (PCI) may come from coadjuvants treatments. Cardioprotective factors applied during revascularization open a new therapeutic window that could increase the clinical ending when it is not possible to modify ischemia duration. The development of strategies to protect the myocardium is based on the understanding of the physiopathological mechanisms of acute cell death during reperfusion. Several of these mechanisms are known; however, many of them are still under investigation. In the last years, various factors implicated with necrosis secondary to reperfusion have been discovered and new potential therapies have been identified. Alternatively, when the myocardium is irreversibly damaged, medical treatment only allows palliative options and effective solution comes from the replacement of the tissue with organ transplantation [7].

The thrilling discovery of stem cells being capable of differentiating into functional target cells and inducing new vascular formations raised worldwide interest and offered new horizons for the patients suffering from cardiac tissue loss. A number of detailed studies focusing on different types of stem and progenitor cells have been carried out in the last decade and cell based therapies have gained acceleration for the regeneration of the injured heart [8] [9]. Up to date, experimental studies indicate the delivery or mobilization of stem and/or progenitor cells may improve tissue perfusion and aid to the functionality of the damaged organs. However, clinical approaches which utilize stem cell transplantation procedures are presently being tested in restricted sets of patients with ischemic heart disease. The ultimate translation of stem cell technologies into clinical practice is expected to be based on treatments with standardized, well characterized cell sources devoid of detrimental tumorigenesis effects, or/and by drugs which rouse endogenous stem cell recruitment for the healing and possible preclusion of cardiovascular disease [10].

2. Acute myocardial infarction

2.1. Scenario

Myocardial infarction is the rapid development of cardiac muscle necrosis caused by a critical imbalance between oxygen supply and demand of the myocardium (ischemia). This situation usually derives from coronary atherosclerosis, a chronic inflammatory disease with stable and unstable periods [11] [12]. During unstable periods, recognized by activated inflammation in the vascular wall, patients may develop a myocardial infarction. Myocardial infarction might be a minor event in a lifelong chronic disease, it may even be undetected, but it may also be a major catastrophic event leading to sudden death or severe hemodynamic deterioration. A myocardial infarction may be the first manifestation of coronary artery disease, or it may occur, repeatedly, in patients with established disease.

MI develops after vulnerable atherosclerotic plaque rupture (unstable collection of lipids i.e. cholesterol and white blood cells, especially macrophages) (Figure 2.1). The rupture, followed by exposure of the basement membrane, triggers to platelet aggregation, thrombus formation, fibrin accumulation, haemorrhage into the plaque and varying degrees of vasospasm. This process can produce a partial or complete occlusion (blockage) of a coronary artery, resulting in an acute reduction of blood supply to a portion of the myocardium (Figure 2.2) located downstream to the blocked vessel (Figure 2.3) [13].

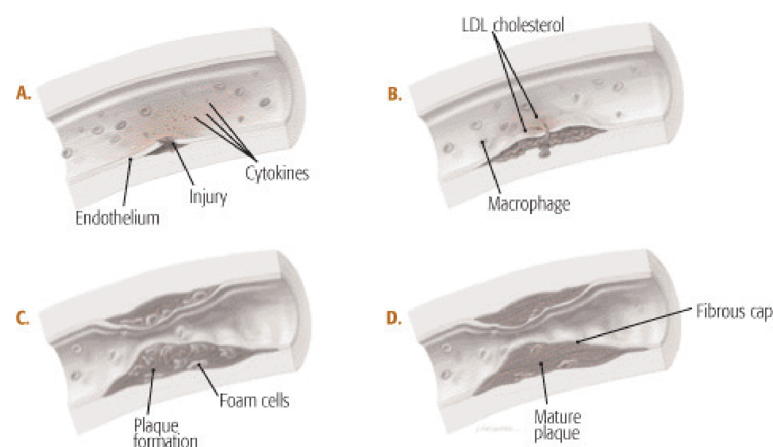


Figure 2.1. Atherosclerotic plaque formation. Image from:

The vulnerability of the plaque depends on its histological structure. Its fragility is related to the size of the lipid core, the thinness of the fibrous capsule and the inflammatory reaction. External aggression favourites rupture. This triggers both thrombogenesis by bringing the blood cells into contact with thrombogenic subendothelial factors and local vasoconstriction due to endothelial dysfunction [13].

Myocardial infarction is defined in pathology as myocardial cell death due to prolonged ischemia. Ischemia describes the acute condition in which blood flow to myocardial cells is not sufficient to meet metabolic demands. Such condition is an unstable state influenced by factors that induce a disproportion between oxygen deliver and oxygen consumption and eventually leads to cell death. The major determinants of myocardial oxygen consumption are the frequency of contraction, the left ventricle wall stress and the inotropic state of the cardiac myocytes [14].

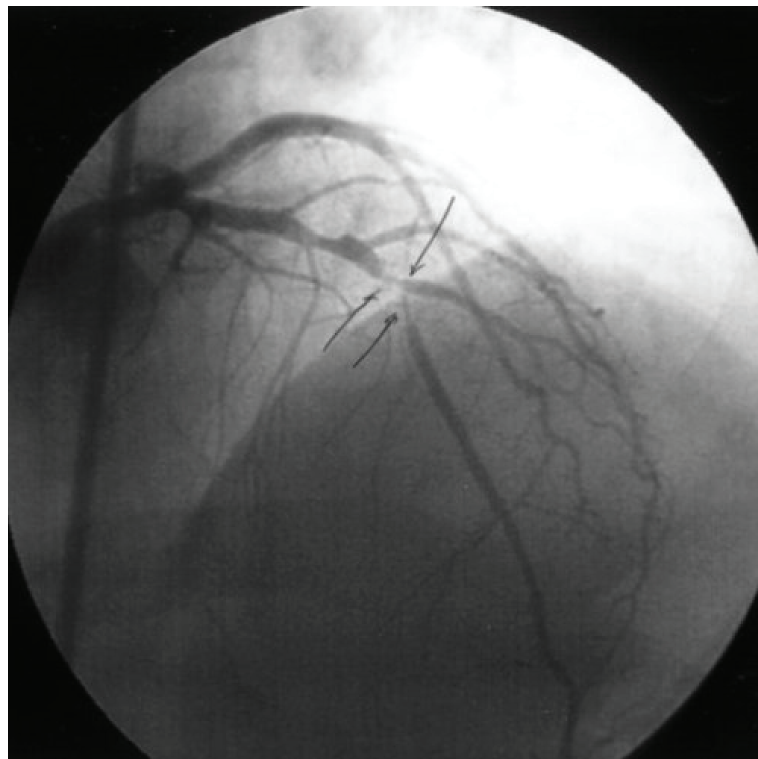


Figure 2.2. Coronary artery blockage. Angiogram from: www.cpiersonmd.com

Although a disproportionate raise in oxygen demand can by itself lead to diffuse and irregular myocardial necrosis, cardiac ischemia in human being is most often regional

and results from an inadequate blood supply trough one of the major coronary artery [15]. The consequences of regional myocardial ischemia have been well studied; anaerobic metabolism and active systolic shortening stop within seconds; when sustained for more than 30 min, ischemia becomes irreversible. Cardiomyocyte death is not immediate but takes a finite period to develop (as little as 20 min or less in some animal models). Cell loss is categorized pathologically as coagulation and/or contraction band necrosis, which usually evolves through oncosis, but can result to a lesser degree from apoptosis.

Before myocardial necrosis can be identified by macroscopic or microscopic post-mortem examination several hours have to pass after ischemia. Complete necrosis of all myocardial cells at risk requires at least 2–4 h or longer (6h) [16]. It depends on: presence of collateral circulation to the ischemic zone, persistent or intermittent coronary arterial occlusion, the sensitivity of the myocytes to ischemia, preconditioning, and/or, finally, individual demand for myocardial oxygen and nutrients [16] [17] [18]. During myocardial infarction development the area of the myocardium is subjected to specific alterations that depend on ischemia distribution. The region is defined as infarcted zone (or infarcted area) while the tissue distant from infarction is recognized as remote zone (or non infarcted area). MI is usually classified by size: microscopic (focal necrosis), small [< 10% of the left ventricular (LV) myocardium], moderate (10–30% of the LV myocardium), and large (>30% of the LV myocardium), and by location [17]. It can be defined pathologically as acute, healing, or healed. Acute MI

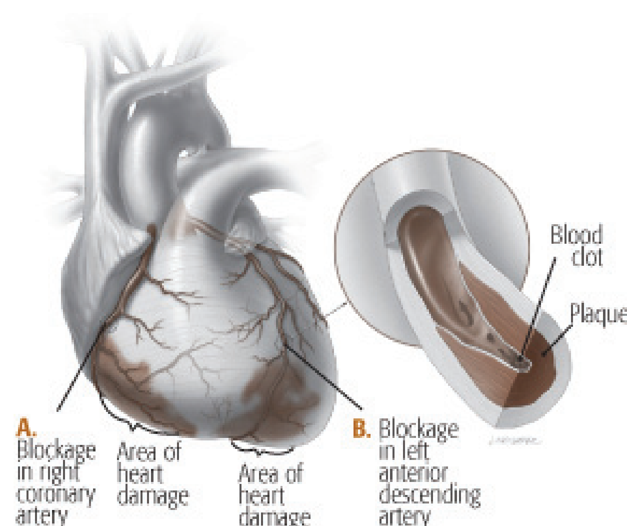


Figure 2.3. A. Right coronary artery blockage. B. Left coronary artery

is characterized by the presence of polymorphonuclear leukocytes. If the time interval between the onset of the infarction and death is quite brief, e.g. 6 h, minimal or no polymorphonuclear leukocytes may be seen. The presence of mononuclear cells and fibroblasts, and the absence of polymorphonuclear leukocytes characterize healing infarction. Healed infarction is manifested as scar tissue without cellular infiltration (Table 2.1). The entire process leading to a healed infarction usually takes at least 5–6 weeks [17].

Types of MI	Presence	Absence
Acute	Polymorphonuclear leukocytes	/
Healing	Mononuclear cells and fibroblasts	Polymorphonuclear leukocytes
Healed	Scar	Mononuclear cells Polymorphonuclear leukocytes

Table 2.1. Myocardial infarction types

2.2. Inflammative response

Inflammatory response and cytokine expression are essential mechanisms of the host response to tissue injury and play a predominant and active role after myocardial infarction (Figure 2.4). The degree of this response is a fundamental aspect of the host's injury overcome and the function of cytokines released by the myocardium is crucial for tissue repair modulation and adaptation to damage [19] [20].

In the first minutes after injury in the ischemic zone there is a significant increase in the production and release of proinflammatory cytokines [21]. This intense cytokines synthesis could control the survival or apoptosis of cardiac myocytes in infarcted area and when extended to noninfarcted zone could activate a second phase of high levels of cytokines that promote interstitial fibrosis and collagen deposition (cardiac remodelling) [22] [23].

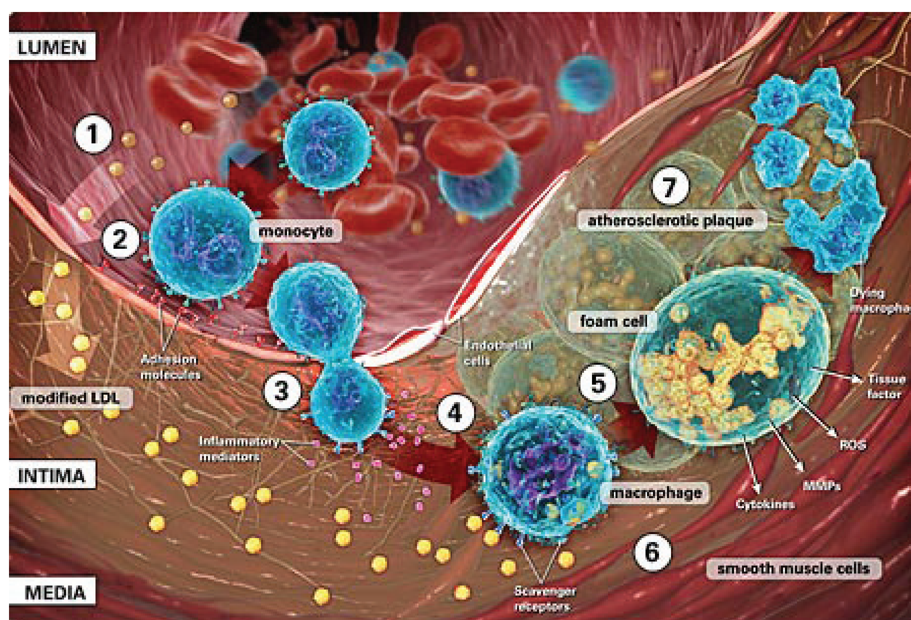


Figure 2.4. Vascular inflammation. 1. Low-density lipoprotein (LDL) extravasation, oxidation and accumulation. 2. Oxidized LDL along stimulate endothelial cells to express adhesion molecules such as VCAM-1, which bind circulating monocytes. 3. Monocytes migrate into the arterial wall, following concentrations of chemoattractants such as MCP-1. 4. In the arterial wall, monocytes mature into activated macrophages, which express scavenger receptors which bind oxidized LDL. 5. Macrophages internalize the oxidized LDL particles giving rise to foam cells. 6. Activated macrophages or foam cells secrete pro-inflammatory cytokines, reactive oxygen species, matrix metalloproteinases and other factors which aggravate the inflammatory process, as well as result in smooth muscle cell proliferation and migration. 7. Foam cells, dead macrophages, lipids and smooth muscle cells accumulate to form a fatty streak, eventually resulting in an atherosclerotic plaque. Image from: www.resverlogix.com

Cytokines can mediate repair through activation of matrix metalloproteinases, regulation of integrins, angiogenesis and progenitor cell mobilization. The early inflammatory reaction can in fact conceal a cardioprotective role that became deleterious when the response is delayed to a stage until fibrosis processes begin. Thus the consequences of inflammatory cytokine effects can be favorable, leading to healing and restoration of function, or unfavorable, leading to acute cardiac rupture or chronic dilatation.

In normal conditions in the heart proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-1-beta (IL-1β) and interleukin-6 (IL-6) are not constitutively expressed [19] [20]. After myocardial injury an intrinsic or an innate stress response mediates upregulation and production of these cytokines [21]. In animal

models (rodents) after MI there are strong upregulations of intramyocardial cytokines including TNF-α, IL-1β, and IL-6 mRNAs in the infarct area, within the first hours to 1 day, (up to 50-fold), as well as in the noninfarcted myocardium (up to 15-fold) [24] [25]. This strong upregulation may return to baseline if the infarction is restricted. However, if the infarction is large, or if host inflammative response is high, there can be either constant cytokine upregulation or a second signal of cytokine overexpression, corresponding to chronic remodeling period [23]. The second signal can expand to involve the remote area with the consequent activation of important remodeling process in the entire myocardium [23] [25].

In the ischemic area during MI, cytokines such as TNF-α and IL-6 are promptly released but normally are found in higher amount in the border zone (area of transition between infarcted and non infarcted area) than in the remote area. Beside the ischemic stress, direct mechanical stretch of the myocardium is also a potent regulator for cytokines production [22]. Mechanical stimulation propagated through potential mechanosensors such as integrins, cytoskeleton and sarcolemmal proteins is translated into 3 major intracellular cross-talking signal transduction pathways [22] [26]. These pathways are: mitogen-activated protein kinase (MAPK), JAK-signal transducer and activator of transcription (STAT), and calcineurin dependent pathways. They trigger related downstream nuclear transcription factors, such as NF-kB and AP-1, which are essential for the induction of most cytokine genes, including TNF-α and IL-6 [26].

The intense upregulation of cytokines after ischemia events is accompanied by a temporary induction of stress-activated transcription factors like C/AATT-enhancer binding protein and STAT-3 phosphorylation [24]. Coupled to this mechanism there is also local gp130 and IL-6 activation which is part of the host stress response signaling system and can direct to phenotype alteration, such as induction of hypertrophy [27] [28]. These signaling pathways which integrate the cell response to stress lead eventually to cytokine activation and are upregulated in reaction to different stimuli like hypoxia, free radical excess, osmotic dysregulation, and early membrane injury.

A new important stress activated inflammatory factor could be the peroxisome proliferator-activated receptor (PPAR-γ). It has been shown that PPARs are regulators of cell proliferation and host inflammatory response. In the murine model the PPAR agonist pioglitazone was found to improve function and remodeling and was associated with significant reductions in inflammatory cytokine levels in the myocardium [29]. An

additional PPAR agonist, rosiglitazone can induce downregulation of CD11b/CD18 and upregulation of cytokines in cardiac ischemia/reperfusion models [30] [31] [32].

Reactive oxygen species (ROS) are also crucial coadjuvant regulators of inflammatory response of myocardium; they are activated by proinflammatory cytokines and they induce cytokines production themselves [33] [34]. A number of studies showed hydrogen peroxide (H_2O_2) can activate TNF-α production via p38 mitogen-activated protein kinase (MAPK) and mediate myocardial dysfunction and apoptosis [33] [35]. Furthermore, H_2O_2 induces the release of the inflammative protein high mobility group box 1 (HMGB1) in macrophages and monocytes [36].

In the heart, known targets of HMGB1 bounds are: TLR 2/4 and advanced glycation endproducts receptor RAGE [37] [38] [39]. Toll like receptors signaling are crucial regulator of cardiac cell survival and myocardial ischemic injury. Beside their pivotal role in host defence against microbial infection (i.e. gram-negative bacteria lipopolysaccharide LPS) and regulation of cardiac dysfunction during sepsis, they can modulate cardiomyocyte survival and ischemic myocardial injury through endogenous ligands recognition [38]. This double-edged effects of TLRs is due to the presence of crosstalk between their signaling and both the cytoprotective (PI3K)/Akt and proinflammatory NF-κB pathways [40] [41] [42]. Moreover, toll like receptors can also mediate induction of ROS after endothelial stress signalling [43]. In recent studies, RAGE has also been shown to be critical for cardiovascular disease and its antagonism has been proposed as novel form of therapeutic intervention [39] [44].

Interestingly, inflammative response is a very dynamic concert of events induced by numerous factors that act in a synergistic way and possess unique self-amplification ability. Cytokines have this self-amplification capacity through a positive feedback mechanism that targets the transcription factor NF-κB. For example, TNF-α upregulation in the ischemic region of the myocardium can easily induce TNF-α upregulation in the near NIZ, initiating amplified cytokine effects [25]. Moreover, the direct recruitment of inflammatory cells to the site of injury produces an additional amplification of inflammative signal. Promptly after cardiac ischemia, adhesive cytokines such as monocyte chemoattractant protein 1 (MCP1), a potent chemoattractant of mononuclear cell, is induced in the myocardium [45]. This gives origin to macrophages transmigration from the blood and, sequentially, supplies a new source of local cytokine production and amplification of the local inflammatory response [46]. The infiltration of neutrophils is another important step in the local

amplification of the initial inflammatory response triggered by cytokines. The migration of neutrophil depends on their interaction with the endothelial cells through L-selectin (on haematopoietic cells side) and P-selectin (on endothelial cells and platelets side) [47]. Leukocyte ß-integrins are, sequentially, responsible for adhesion to endothelial cells, especially the ß2 integrins (CD18), lymphocyte function antigen-1 (CD11a/CD18), macrophage antigen-1 (CD11b/CD18), or the very late antigen-4 (CD49d/CD29). Once the transmigration of inflammative cell takes place, further cytokines are secreted to facilitate extravasation into the extravascular environment [47].

Beside the role of mononuclear cells such as macrophages and neutrophils in the early inflammative response, other cells like e.g. mast cells hold an important function. These cells are bone marrow-derived inflammatory cells with prepackaged inflammatory cytokines and growth factors and accumulate in the ischemic-reperfused myocardium after 3 days from infarction. Mast cells could principally follow the mobilization signal of stem cell factor (SCF) which is secreted by a macrophage subset during reperfusion. In the early inflammatory response, mast cell release as well TNF-α which can further amplify inflammation cascade [48].

2.3. Ischemia/reperfusion injury

After ischemia the coronary artery stenosis (culprit lesion) has to be reopened or bypassed early enough to attenuate myocardial infarction. When the reperfusion begins, the cells that are able to re-acquire the ionic homeostasis survive, while in a variable number of cardiac myocytes the ionic unbalance is not restored but even deteriorated. The unavoidable fate of these cells is the sudden death for necrosis. This adverse situation happens during the first minutes after oxygen and blood supply restoration and is characterized by cellular membranes rupture and liberation of cellular content (principally cytosolic enzymes) in the extracellular milieu [49]. Necrosis is well defined in histological samples where single cardiac myocytes appear significantly shorter with complete loss of sarcomeric organization which leads to the characteristic histological features of contraction bands (Figure 2.5). Electronic microscopy images of cardiac muscle cells show sarcolemma disaggregation, mitochondrial edema, Ca^{2+} massive deposits in mitochondrial matrix and sarcomeric myofibrils shortening and disorganization [50].

A classic laboratory experiment which could mimic adequately the features of this necrosis is the retrograde heart reperfusion by the Langendorff system. A transient ischemia (normally between 40 and 60 min) and following reperfusion are artificially produced in the isolated heart. The first minutes of reperfusion are characterized by a wide release of intracellular enzymes (creatine kinase, lactate dehydrogenase) which amount correlates with the extension of the necrotic tissue detected by the typical contraction bands in histological sections. These findings demonstrated that the myocardial cell death happens promptly at the moment of flow restoration [51].

Other studies focused on isolated cardiomyocytes models proved that reoxygenation after artificial ischemia induces a sudden cell length shortening accompanied by the loss of the cytoarchitecture. The ultrastructural features of this abrupt cell disorganization also coincide with the typical traits of necrosis (contraction bands) [52]. The typology of response at cellular level is denominated hypercontracture and its occurrence depends on the time frame between severe depletion of adenosine-5'-triphosphate (ATP) and reenergization that follow reperfusion [53]. The amplitude of cardiac myocyte shortening has been shown to correlate again to the extension of contraction bands area [54].

The mechanisms that lead to cellular shortening due to hypercontracture have been widely investigated with isolated cardiac myocyte models. These models are especially suitable for the simultaneous analysis of morphological changes and ionic unbalance. The studies highlighted that hypercontracture is mainly caused by reenergization (which reactivates contractile activity of ATP dependent myofibrils) and the concurrent presence of anomalous elevation of intracellular Ca^{2+} (that in presence of ATP produce uncontrolled and excessive contractile power) [55]. The loss of Ca^{2+} homeostasis begins in the very early instants of ischemia characterized by determinant changes in the cytosolic composition [53]. The initial ionic unbalance originated by energy deficiency is the abnormal accumulation of intracellular Na^{+} due to sarcolemmal Na^{+}/K^{+} pump failure and cytosolic acidity increase induced by initiation of anaerobic glycolysis. This ionic unbalance gives origin to a reaction of the cell that try to re-stabilize the internal milieu by the inverse flow of Na^{+}/Ca^{2+} membrane exchanger with unavoidable Ca^{2+} uptake; the condition leads to progressive loss of Ca^{2+} concentration control. In normal physiological conditions, the Ca^{2+} is one of the cations which concentration is more strictly regulated inside the intracellular environment [50].

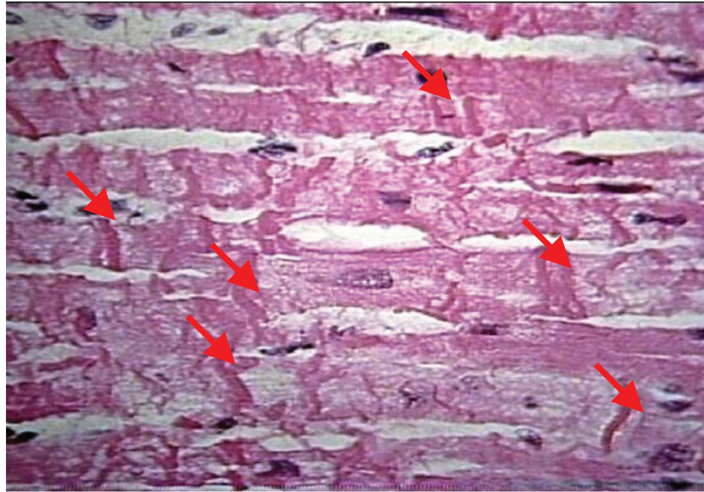


Figure 2.5. Contraction bands. Image from: www.pathguy.com

At this point, the abrupt reperfusion and reenergization leads to an aggravation of the cations control associated to the cell mechanisms for acidosis regulation. The blood reflow provokes a fast washing of extracellular catabolites (mostly H^+) which induces a pH gradient between the cell and its surrounding causing activation of regulatory mechanisms against intracellular acidosis. These mechanisms, mainly through trans-membrane exchanger Na^+/H^+ and co-transporter $Na^+/HCO3^-$, further exacerbate the cytosolic overcharge of Na^+ that as previously mentioned contribute to Ca^{2+} uncontrolled increase [56]. Under cardiac myocyte normal physiological conditions, the entrance of Ca^{2+} through inverse flow of Na^+/Ca^{2+} exchanger is not relevant but became deleterious when the cell is overcharged by Na^+. As a result of all these chained regulatory mechanisms, the re-oxygenated cell accumulates an amount of intracellular Ca^{2+} that seriously compromises its survival [56].

Beside the critical ionic unbalance in the intracellular environment, the reactivation of the energetic metabolism holds direct consequences on the integrity and the functionality of vital cellular structures such as cytoskeleton, organelles and sarcolemma. The first aspect is cellular edema induced by the osmotic gradient through the sarcolemma that suddenly forms, following metabolites washing. This promotes H_2O entry in the cytosol with consequent cell volume augmentation, cytoskeleton and membrane stress and eventually cell integrity loss. Experimental studies demonstrated that the heart when reperfused with an hyperosmotic solution shows decreased: infarction size, edema extent and cellular death [57]. The second unfavorable aspect is

that the developing of mechanic fragility during ischemia can significantly reduce the cell resistance to the mechanical stress imposed by reperfusion. The actual mechanisms which lead to cellular structures vulnerability are not fully understood, but it has been described that the activation of calpains could provoke the proteolysis of cytoskeleton and subsarcolemmal structures [58] [59]. Calpains are a family of calcium-dependent, non-lysosomal cysteine proteases (proteolytic enzymes) that are inhibited by acidosis. However, these proteases play a crucial role in the death of cardiac myocytes during reperfusion because of antagonism between Ca^{2+} overcharge and pH normalization. Calpain mediates degradation of ankyrin (a protein which participates to the attachment of the Na^{+}/ K^{+} ATPase pump to the membrane and subsarcolemmal cytoskeleton) causing decisive dysfunction of the pump during initial reperfusion [60]. This proteolytic degradation could create a close circle in which Na^{+} overcharge promotes additional Ca^{2+} inflow that holds calpains activated state which additionally increases intracellular Na^{+} and finally leads to hypercontracture and cellular death [50].

Finally it is important to remember that although the majority of the necrosis features secondary to ischemia are reproducible with the preparation of isolated cardiac myocytes and perfused hearts, other cell types such as thrombocytes, neutrophils and fibroblasts play a trivial role in reperfusion damages. Particularly platelets activated during ischemia/reperfusion adhere to myocardial microvascular endothelium through L-selectin induction and secrete factors which contribute to the Ca^{2+} homeostasis loss and consequent cell death [61] [62] [63]. Additionally, the activation of NF-κB pathways has been shown to be crucial as well in the dynamic of reperfusion injury and its inhibition can alleviate the damage of sudden blood reflow [64] [65] [66].

2.4. Cardiac remodeling

The cardiac remodeling is the progressive and detrimental alteration of the myocardium in response to injury initiated by succeeding inflammatory cascade phases that ultimately results in the formation of a collagen-based scar [67]. Particularly, the ventricular remodeling involves both the infarcted and noninfarcted myocardium and results in dilation, hypertrophy, and enhanced sphericity of the ventricle [68]. This process, characterized by changes in size, shape and functions is directly related to the healing response and is associated with a worse prognosis in patients with heart failure [69] [70].

Pathologically, the scar formation is well discriminated by the infarct size [71]. Its extension depends on: the area of myocardium interested by ischemia, the reperfusion therapy time frame, and the myocardial energy consumption during coronary occlusion. The injured area of the heart after reperfusion consists of highly inhomogeneous tissue which could either recuperate, persist in shock state or became apoptotic / necrotic. The complexity of this irregular situation is enlarged by the different sensitivity to ischemia of myocardial cells, vasculature, and connective tissue [72] and will be even more intricate when inflammatory cells and macrophages invade the injured area and start to support processes of inflammation, clearing debris, and wound healing [73].

The whole course of events is initially dominated by the inflammation phase (degradation of extracellular matrix, inhibition of tissue proliferation, and release of inflammatory mediators. Defined also as: ‘acute remodeling’ or ‘inflammatory phenotype’) and after evolve to reparation (increased matrix synthesis, proliferation of fibroblasts and inflammatory cells, and release of fibrosis-promoting cytokines leading to scar formation. Defined also as: ‘chronic remodeling’ or ‘activated phenotype’) [73].

As described before, crucial factors involved in the initial inflammation phase (injury response and acute remodeling) such TNF-α, IL-1β, and IL-6 are most commonly associated with the postmyocardial infarction remodeling process as well (chronic remodeling) [25] [23]. In the acute remodelling phase, these proinflammatory cytokines have at least four direct effects on cardiac myocytes: progressive myocyte apoptosis [74] [75], myocyte hypertrophy [76], defects in contractility [77] and inflammatory signal transduction [78]. Regarding myocyte apoptosis and hypertrophy, has been shown that cytokines like TNF-α or IL-6 showed to have a significant pleiotropic effect on the host cells, with a potential for apoptosis versus cytoprotection and hypertrophy [76] [77] [79] [80]. The balance between these opposite process rules the cellular remodeling degree. Cytokines are able to reduce left ventricle performance and myocyte contractility directly and indirectly. TNF-α and IL-6 can directly ease the contractility of cardiomyocyte by alterations of sarcoplasmic reticulum function with the instant reduction of systolic cytosolic Ca^{2+} [81]. On the other hand, TNF-α is also capable of diminishing myocyte contractility indirectly, through nitric oxide-dependent decrease of myofilament Ca^{2+} sensitivity [82]. Contract failure could be also induced by TNF-α, IL-1β, and interferon-γ through the raise of superoxide anion, which reacts with NO to form peroxynitrite which in turn desensitizes myofilaments [83]. During damage,

the fall of contractility mediated by TNF-α and IL-6 might be an adaptive response to the reduction of cardiac energy demand.

TNF-α could also directly stimulate mitochondrial ROS production within cardiac myocytes and caused mitochondrial DNA damage via a ceramide-dependent pathway [84]. ROS, on its side, has also been shown to participate to cardiac myocyte hypertrophy progress. Furthermore, ROS release is involved in the successive chronic remodeling processes including fibrosis, collagen deposition, and matrix metalloproteinase activation which are mainly associated with pathological fibrosis, feature of development toward end-stage heart failure [85] [86] [87].

The wound healing process (chronic remodeling) includes: phagocytosis and removal of necrotic tissue, myocytes survival and hypertrophy, degradation and synthesis of matrix substrate such as collagens and integrins, myofibroblasts proliferation and angiogenesis / vasculogenesis, and, to a limited extent, progenitor cell proliferation [88] [89] [90].

After the first upregulation of proinflammatory cytokines in the infarcted area the level of TNF-α, IL-1β, and IL-6 should decrease to the baseline within one week [23]. However, depending on infarct dimensions and potential additional stress signal the cytokines could either persist at a high level of expression or grow back to a second wave of upregulation especially in the remote area, far from the origin of the injury. During progression of chronic remodeling, cytokine such as IL-1β are originated in macrophages, endothelial cells, and vascular smooth muscle cells in addition to myocytes. These cytokine mediates processes of myocyte hypertrophy, myocyte apoptosis, and the triggering of additional inflammatory cell signaling. Several studies confirmed these effects by a number of transgenic mice models with myocardial TNF-α overexpression [77] [91] [92]. These studies consistently showed myocardial hypertrophy, eventually leading to dilated cardiomyopathy, inflammatory cell infiltrations, and greater interstitial fibrosis.

After the resorption of necrotic tissue and the removal of apoptotic granulocytes by phagocytes the proinflammatory cytokines release decreases. In fact, apoptosis in contrast to necrosis (necrotic cells triggers inflammation) promotes the production of anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF). The latter is a central cytokine for the transition phase from inflammation to fibrosis, it decreases leukocyte adhesion and stimulates fibroblast proliferation and extracellular matrix production [93]. Cytokines elevation (e.g. TGF elevation) could promote

interstitial fibrosis and collagen deposition not only in the infarcted region but in the contralateral remote area as well. These remodeling mechanisms are characterized mainly by the synthesis and degradation of collagens which are the most important components of the extracellular matrix. The mediators of this matrix remodeling are the matrix metalloproteinases (MMPs) that reside usually in the myocardial interstitium under inactive form; MMPs can be rapidly triggered by free radicals, cytokines, and hypoxia after few minutes of ischemia [94]. Within these cytokines, TNF-α and IL-1β can regulate the activation and overexpression of MMPs that are originally responsible for matrix degradation and, consequently, collagen deposition [95] [96].

Beside collagens, other proteins such as elastin and fibronectin constitute the intricate network which composes the cardiac matrix; these proteins interact with integrins and adhesion kinases at the cell / matrix connection. Even so, during the remodeling phase the most prevalent protein alteration in the myocardium consists of collagen degradation and production. The cytokines that play a major role in this process are TNF-α, TGF-β and osteopontin. The increase of such cytokines correlates closely with the consequent deposition of collagens type I and III. It has been shown that TNF-α could enhance fibrosis through angiotensin 1 (AT1) receptor upregulation [97] while TGF-β3 might be implicated in the regulation of late collagen deposition [88]. Osteopontin (OPN) plays an important role in myocardial post infarction remodeling by inducing collagen synthesis and accumulation [98] [99].

Interestingly, TNF-α is also capable to alterate the integrin-β1 form present in the adult myocytes (integrin-β1D, responsible for the firm anchoring of myocytes in the matrix) to the isoform integrin-β1A, which is present in the fetal myocytes and promotes mobility and proliferation at the expense of efficient contractility [90].

The remodeling process is not only characterized by collagen deposition and scar formation with subsequent worse ventricular modification but also by a certain degree of tissue restoration. Indeed, part of the alteration postmyocardial infarction involves the regeneration of some of the lost cardiac components like blood vessels and myocyte. The prior knowledge of the heart being an organ incapable of self-regeneration has been recently overturned by evidence suggesting the myocardium could partially restore some of these components and that this partial regeneration could be also enhanced [89] [attached manuscripts 1 and 2] [100] [101] [102].

Important cytokines elaborated during myocardial infarction have been repeatedly shown to direct the angiogenesis process. In the initial phase, the expression levels of

these cytokines such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor bFGF, angiopoietin-2 (Ang-2), hepatocyte growth factor (HGF), transforming growth factor (TGF)-beta1, inducible nitric oxide synthases (iNOS) and TNF-α are significantly elevated [103] [104] [105]. Some of these factors (VEGF, iNOS and TNF-α) have been shown to remain elevated within one month or more [103] or to be subjected to a second wave of up-regulation after the acute phase (during the sub-acute phase) [105].

Other angiogenetic mediators are the chemotactic cytokines (chemokines) of the "CXC" family. Within these cytokines, the ones that contain the so called ELR motif (glutamic acid-leucine-arginine) like interleukin 8 (IL-8) are angiogenesis inducers while the ones lacking ELR motif such as interferon-inducible protein 10 (IP-10) are angiogenesis inhibitors [22].

In the very first hours of reperfusion, the upregulation of TNF-α could induce synthesis of IP-10 in the microvascular endothelium. IP-10 overexpression provokes inhibition of angiogenetic activity in the injured area until macrophages ultimate complete remove of death cells and debris and fibrin-based provisional matrix is formed. After the first 24 hours of reperfusion, under TGF-β1 stimuli IP-10 is downregulated again in favor of angiogenesis. TGF-β can also induce bFGF and VEGF significant overexpression in endothelial and smooth muscle cells amplifying angiogenetic progression. Together with endothelial and smooth muscle cells monocyte-derived macrophages, mast cells, and myofibroblasts secrete essential growth factors for new vessel formation and repair [22].

2.5. Therapies for acute myocardial infarction

Usual treatments to counter coronary occlusion in patients with acute myocardial infarction (MI) are attempted by the redirection of the blood flow (reperfusion therapy). The reperfusion therapy has become the central treatment for patients who present with suspected acute MI and can be achieved either with thrombolytic therapy, percutaneous coronary intervention (PCI) or when these therapies are unsuccessful, bypass surgery.

In the favorable case the patient can be treated in the first hours from symptoms presentation, one possible intervention to restore perfusion is the thrombolytic therapy. The efficiency of this therapy is maximal within 2 hours from the manifestation of the symptoms and must be performed in any case before 12 hours from adverse event. In

fact, away from this intervention window the administration of thrombolytic agents causes more risks (e.g. intracranial bleeding) than effective benefits [106] [107]. As previously mentioned, irreversible injury occurs within 2–6 hours from the infarction. Therefore the therapeutic window to re-establish perfusion is extremely narrow. Beside a rapid administration, an ideal thrombolytic drug should direct to quick reperfusion, be specific for recent thrombi, create a low risk for intra-cerebral and systemic bleeding, have no antigenicity, adverse hemodynamic effects, or clinically significant drug interactions [108]. Currently, no perfect thrombolytic agent exists and thrombolytic therapy could be often ineffective with a degree of success that depends on the time since the onset of symptoms began [107]. The failure rates of thrombolytics can be as high as 20% or higher [109].

Nowadays, thrombolysis should only be performed if it is not possible to transport the patient to the nearest cardiovascular centre within 90 minutes from MI symptoms presentation. In the adverse cases of thrombolytic agent inefficiency, the patient could be then treated with percutaneous coronary intervention (PCI). Percutaneous coronary intervention following thrombolytic treatment is defined as "rescue PCI" or "salvage PCI". Due to the action of the thrombolytic agent, a number of problems (particularly bleeding) are significantly higher with rescue PCI than with primary PCI [110].

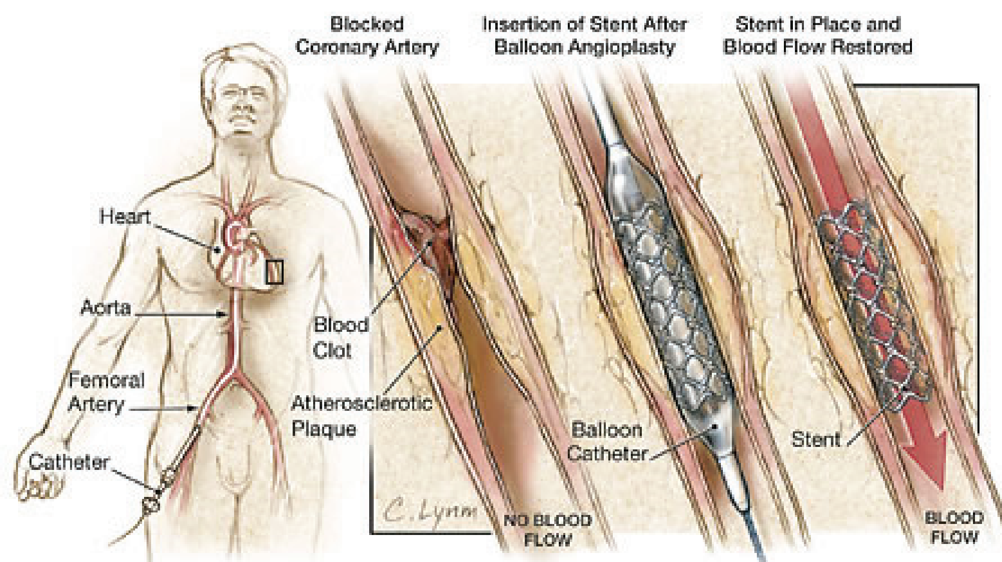
Primary percutaneous coronary intervention is a well established and firs line therapy for acute ST elevation myocardial infarction [111] [112] [113]. Primary PCI consists of anatomical localization of the occluded vessel by coronary angiogram followed by balloon angioplasty through femoral or radial artery (and commonly employment of an intracoronary stent) (Figure 2.6). In some cases, an attempt to remove the thrombus using extraction catheter might be tried prior to balloon angioplasty [114]. Properly performed primary PCI restores flow in the offended artery in more than 95% of patients compared with the spontaneous recanalization rate of about 65% [111].

Reperfusion therapy obtained by emergency coronary artery bypass graft surgery (CABG) to treat acute MI is less common than PCI or other medications (U.S. National Registry of Myocardial Infarction) [115]. Coronary artery bypass surgery is characterized by the implantation of an artery or a vein from the patient to bypass narrowing or occlusions of coronary arteries. Different vessels could be utilized, however internal mammary artery grafts have shown considerably better long-term patency rates than great saphenous vein grafts [116]. In the case more than one coronary

artery is completely or partially occluded, bypass surgery is associated with higher long-term survival rates compared to percutaneous interventions [117]. If the vessel affected is only one, surgery and PCI have comparable safety and efficiency and selection of intervention type depends on specific cases [118].

In the last years, new therapies for the treatment of myocardial infarction rose consistently following the thrilling advances in stem cell biology. On the basis of encouraging results in animal models, a number of clinical trials with the application of stem cells after MI have been carried on. Patients who received stem cells derived from different sources through coronary artery injection or other transplantation routes showed improvements in left ventricular ejection fraction and end-diastolic volume not seen with placebo [119] [120]. Several clinical trials with stem cell application are still proceeding and stimulating optimism about the prevention or even reversion of heart failure by cell-based therapy.

Additional approaches which are still at an earlier stage of medical research consider biomaterial and tissue engineering for the treatment of myocardial infarction. Special polymers, employed as left ventricular scaffolds, and in vitro engineered cardiac tissue, which is subsequently implanted in vivo are investigated in order to prevent heart failure [121].



2.6. What to target?

In a complex scenario like the myocardial infarction, it is particularly challenging to understand what could be the best move towards the preservation and/or renovation of the cardiac tissue. Within the multifaceted mechanisms that regulate the inflammative response, the reaction to reperfusion and the remodeling after and during cardiac injury, the best intervention might be not exclusive but most likely a concert of actions. Although no perfect cure exists, the optimal treatment should: reduce inflammation, protect the cells from mechanical and oxidative stress, decrease apoptosis, necrosis and fibrosis, and initiate regeneration processes directly and through innate renewal activation. The finding of stem cells being capable of several of these beneficial actions at a time (e.g. immunosuppressant properties, cytoprotection, direct regeneration through transdifferentiation and activation of resident stem cells) [120] opened an exciting possibility for many scientists to aspire at the ideal treatment for myocardial infarction and other diseases.

3. Stem cell mediated cardiac repair

3.1. Cardiac regeneration

The dogma of the heart being a post-mitotic organ formed by cardiac myocytes (often compared to neurons) which are incapable of regeneration and replacement of the damaged areas of the myocardium has recently been overcome [120] [122]. The concept of cardiac myocyte regeneration took some time to be embraced by the medical community and it remained highly disputed [89]. Currently, only few queries in cardiac regeneration are definitely resolved but it is widely accepted that the cardiac tissue holds a certain degree of restoration capacity. Nevertheless, this innate renewal ability of the heart is far to be sufficient to compensate the severe loss of cardiac muscle which appears after catastrophic events such as myocardial infarction or other cardiac diseases [120].

In contrast to the heart, the skeletal muscle (in mammals) is capable of efficient regenerative processes even after extensive insult [123] [124]. A large number of new myotubes is build by satellite cells and other types of myoblasts after few days from muscle injury. In the heart, a regenerative response after damage is well documented only in some vertebrates such as zebrafish and newts [125] [126]. In newts, cardiac myocytes undergo to mitosis very seldom in normal conditions but they re-enter the cell cycle as soon as an injury appears. In this species, the dedifferentiation and subsequent division of existing cardiomyocytes seems to be the central dynamic of cardiac regeneration [125].

In other vertebrates such as zebra fish, cardiac regeneration may be due mainly to activation and differentiation of undifferentiated stem or progenitor cells localized in the epicardial stratum of the heart [127]. On the contrary, in mammalian hearts, cardiac myocytes divide extensively only during foetal development and stop their cell cycling shortly after birth [128]. In fact in the adult mammals, cardiomyocytes proliferate very seldom after a serious injury, even if located at the border zone of the infarction [129] [130]. On the other hand, it has been shown in mice that transgenic overexpression of certain genes or repression of cell cycle inhibitors such as p38 MAP kinase can enhance cardiomyocytes proliferation [130] [131].

A much more significant renewal of the heart tissue comes from endothelial cells. It has been strongly demonstrated that bone marrow derived progenitor cells are capable

to give origin to mature endothelial cells but their ability to generate cardiomyocytes is still disputed [132]. In contrast, several investigators have now confirmed the existence in the mammalian myocardium of a population of resident cardiac stem cells (CSCs) which are able to differentiate into cardiac myocytes or other cell types such as endothelial and vascular smooth muscle cell [133] [134] [135]. CSCs have been proposed to maintain the cardiomyocyte basal turnover [125] [132]; however this mechanism takes place at a very low rate if there is no presence of injury [136].

The understanding that the mammalian heart is also interested by regenerative processes brings now the attention into the definition of the obstacles which could prevent regeneration, including inflammation, ischemia and remodelling (fibrosis) (Figure 3.1) [120]. The adverse microenvironment present into the insulted myocardium could counteract the desirable activity of both resident (CSCs) and exogenous stem cells (e.g. stem cell transplantation therapies).

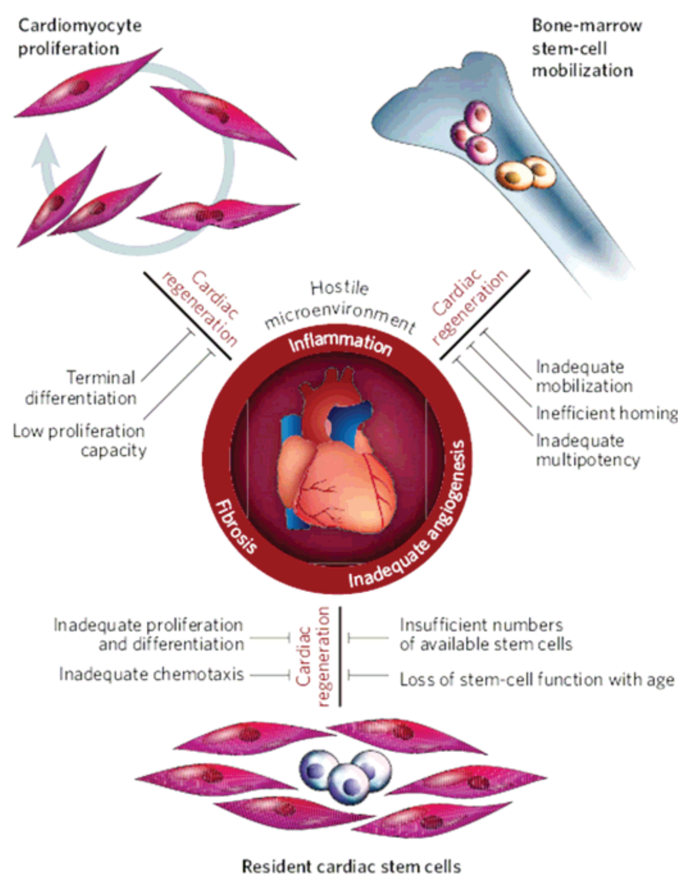


Figure 3.1. Potential barriers to cardiac regeneration [120].

The response to injury corresponds to the activation of a concert of mechanisms that are definitely beneficial to some extent but become deleterious after a certain degree (e.g. inflammatory reaction that on one side promotes angiogenesis and progenitor cell recruitment and on the other prevents recruitment and survival of progenitor cells; or: protective mechanism of fibrosis against myocardium rupture and its action as very strong barrier to regenerating cells) [137]. A balance between beneficial and deleterious cardiac reaction to damage might favour revascularization and cell regeneration instead of apoptosis and fibrosis. A step forward to artificially control those optimal conditions for cardiac regeneration during response to injury would open novel prospects and direct more efficient stem cell therapies.

3.2. Stem cell therapies and stem cell sources

During the past decade, several sources of stem cells have been considered as suitable candidate for cardiac repair and numerous transplantation routes have been used [120] [attached manuscript 3]. The ideal cell type or the best transplantation route have not been clarified yet and the complete understanding of mechanisms at different levels is far to be accomplished. However, on the wave of promising results obtained with the animal models a number of clinical trial has been carried on in the last seven years (Table 3.1) [120].

Initially, autologous skeletal myoblasts were considered as possible candidate for cardiac regeneration and the cells were injected directly into the ischemic myocardium [138]. Although these cells differentiate into myotubes *in vivo* and improve ventricular functions in the animal model [138], they do not give origin to cardiomyocytes and do not integrate electrically with the surrounding cells [132]. Thus, they do not beat in synchrony with the myocardium and they lead to sustained arrhythmia. Clinical trials with skeletal myoblasts are ongoing but some have been stopped because of treatment inefficacy [139].

More successful source of cells for cardiac regeneration than skeletal myoblasts has come from the bone marrow. In fact, bone marrow derived haematopoietic cells (HSC) have been the first cells shown to differentiate into cardiac myocytes after transplantation in the mouse myocardium [140] [141]. Additional studies demonstrated direct differentiation of haematopoietic stem cells into cardiac myocytes after bone marrow transplantation and myocardial infarction; however the labelled cells that

originated new cardiomyocytes were extremely few [142]. In contrast, several experiments showed complete absence of haematopoietic stem cell differentiation into cardiac myocytes [143] [144] or ventricular function improvement [145].

Cell type	N. of patient	Follow-up (months)	Number of cells injected	Route of injection	Ejectionfraction vs control (%)†	Source
BMMNC	60	12	10^{8}	Intracoronary	+7.0 ($P = 0.03$)	Meluzin *et al*. (2007)
	51	3	$2 . 10^{8}$	Intracoronary	+4.1 ($P = 0.001$)	Assmus *et al*. (2006)
	66	3	10^{8}	Intracoronary	+3 ($P = 0.04$)	Meluzin *et al*. (2006)
	204	12	$2.4 . 10^{8}$	Intracoronary	Decreased mortality	Schächinger *et al*. (2006)
	20	6	$4 . 10^{7}$	Intracoronary	+6.7 (NS)	Ge *et al*. (2006)
	20	4	$6 . 10^{7}$	TEIM	+2.5 (NS)	Hendrikx *et al*. (2006)
	67	4	$1.7 . 10^{8}$	Intracoronary	+1.2 (NS)	Janssens *et al*. (2006)
	100	6	$8.7 . 10^{7}$	Intracoronary	–3.0 ($P = 0.05$)	Lunde *et al*. (2006)
	60	18	$2.5 . 10^{9}$	Intracoronary	+2.8 (NS)	Meyer *et al*. (2006)
	36	3	$3 . 10^{8}$	TEIM	+4.0 (NS)	Mocini *et al*. (2006)
	204	4	$2.4 . 10^{8}$	Intracoronary	+2.5 ($P = 0.01$)	Schächinger *et al*. (2006)
	36	3	$9 . 10^{7}$	Intracoronary	+7.0 ($P = 0.02$)	Strauer *et al*. (2005)
	20	12	$2.6 . 10^{7}$	TEIM	+8.1 (NS)	Perin *et al*. (2004)
	20	3	$2.8 . 10^{7}$	Intracoronary	+1.0 (NS)	Strauer *et al*. (2002)
CPC	54	6	$5 . 10^{9}$	Intracoronary	+6.0 ($P = 0.04$)	Tatsumi *et al*. (2007)
	73	6	$2 . 10^{9}$	Intracoronary	+2.8 (NS)	Choi *et al*. (2007)
	47	3	$2 . 10^{7}$	Intracoronary	+0.8 (NS)	Assmus *et al*. (2006)
	82	6	$1.4 . 10^{9}$	Intracoronary	–0.2 (NS)	Kang *et al*. (2006)
	70	6	$7.3 . 10^{7}$	Intracoronary	+5.5 ($P = 0.04$)	Li *et al*. (2006)
	26	3	$7 . 10^{7}$	Intracoronary	+7.2 (NS)	Erbs *et al*. (2005)
CD133+	27	6	NA	Intramyocardial‖	NA	Ahmadi *et al*. (2007)
	55	6	$6 . 10^{6}$	Intramyocardial‖	+6.3 ($P = 0.02$)	Stamm *et al*. (2007)
	35	4	$1.3 . 10^{7}$	Intracoronary	+2.8 (NS)	Bartunek *et al*. (2005)
CD34+	24	6	$3.5 . 10^{7}$	TEIM	NA	Losordo *et al*. (2007)
SMB	97	6	NA	Intramyocardial‖	+3 ($P < 0.04$)	MAGIC (2007)
	26	12	$2.5 . 10^{8}$	Intramyocardial‖	+14.5 ($P < 0.01$)	Gavira *et al*. (2006)
	12	12	$2.1 . 10^{8}$	TEIM	+11.6 ($P < 0.05$)	Ince *et al*. (2004)
MSC	48	12	$5 . 10^{6}$	Intracoronary	–3 (NS)	Chen *et al*. (2006)
	69	6	$6 . 10^{10}$	Intracoronary	+12.0 ($P = 0.01$)	Chen *et al*. (2004)
MSC +	22	4	$3 . 10^{6}$	Intracoronary	+0.3 (NS)	Katritsis *et al*. (2005)
BMC	20	6	NA	Intracoronary	+9.2 ($P < 0.05$)	Ruan *et al*. (2005)

Table 3.1. Overview of clinical trials of stem-cell or progenitor-cell delivery to the heart.
BMC, bone-marrow-derived cells (unspecified); BMMNC, bone-marrow mononuclear cell; CPC, circulating progenitor cell; DB, EPC, endothelial progenitor cell; MSC, mesenchymal stem cell; NA, not available; NS, not significant; SMB, skeletal myoblast; TEIM, transendocardial intramyocardial injection. *The number of patients is the sum of individuals in the control and treatment groups; almost all studies have equal numbers in each group. †Ejection fraction is the proportion of blood in the left ventricle that is ejected into the aorta during each heartbeat; this is a measure of cardiac function. ‖Intramyocardial indicates injection through the epicardial side of the heart.

Nowadays, the effective ability of these cells to derive cardiomyocytes *in vivo* is still disputed. On the other hand, it is well accepted that a subset of bone marrow haematopoietic cells defined as endothelial progenitor cells (EPCs) have the potential to originate endothelial cells both *in vitro* and *in vivo* (often identified as $CD133^{+}VEGFR2^{+}$) [146] [147]. Most likely, EPCs do not contribute to direct differentiation into cardiomyocytes *in vivo* but they have a strong direct angiogenetic potential [130] [146] and a cytoprotective role for the cardiac myocytes via paracrine signals [148]. Endothelial progenitors can be readily isolated from blood and bone marrow and several studies propose they improve myocardial functions [4] [5] [149] [150]. However, the characterization of EPCs is far to have a general consensus thus it is still difficult to compare different studies in which different types of cells were probably used [151].

Beside the haematopoietic tissue in the bone marrow, an additional source of multipotent stem cell resides in the supporting stroma and it is identified as mesenchymal stem cells (MSCs) [152]. MSCs are known to differentiate in osteoblasts, adipocytes and chondrocytes [153] but only a subset of mesenchymal stem cell population can differentiate into cardiomyocytes or neurons under definite conditions *in vitro* [8] [152] [153]. Mesenchymal stem cells have been shown to differentiate into cardiomyocytes *in vivo* as well, although at a very low rate [154] [155].

The potential advantages of MSCs are diverse: first of all, these cells can be readily separated from the haematopoietic compartment of the bone marrow and expanded *in vitro* for several passages (even 25, which correspond to 50 cell divisions). Therefore, from a limited amount of starting material such as 1 mil of human bone marrow aspirate, billions of mesenchymal stem cells could be obtained [152]. In addition, MSCs are known to be significantly less immunogenic than other stem cells, permitting transplantation of allogeneic cells [155]. Moreover, MSCs are widely accepted to hold a strong beneficial paracrine effect, which could support and preserve the other cells present in the injured myocardium [153] [156]. Finally, it seems possible to further increase the MSCs therapeutic potential using gene modification approach in order to induce the overexpression of prosurvival, angiogenic, growth or stem cell homing factors [102] [153].

MSCs present some disadvantages as well: for example, the differentiation behaviour of mesenchymal stem cells *in vivo* seems to be uncorrelated with their differentiation capacity *in vitro* creating several difficulties in the selection of the

optimal population for transplant after manipulation [152]. Additional problems rise from the uncontrolled differentiation capacity which could lead to undesirable cell formations; indeed, MSCs have been found to give origin to bone-forming osteoblasts after transplantation in the mouse heart [7]. Moreover, it has been described that artificial cell expansion may switch deleterious mutation which could result in spontaneous immortalization and malignant transformation of adipose tissue-derived human MSCs [157], bone marrow-derived mouse MSCs [158] [159] [160] [161] and recently bone marrow derived rat MSCs [attached manuscript 4]. These results recall the attention on the importance of preventing mesenchymal stem cells from unrestrained differentiation or malignant transformation with consequent undesirable potential of sarcoma formation [120] [160].

A different source of stem cell that holds high consideration in regenerative medicine worldwide is the embryonic stem (ES) cell. Due to its characteristics, the embryonic stem cell seems to be the prototype of all stem cells; in fact, it has high clonality potential, self renewal capacity and is totipotent [162] [163]. All together these features make ES cell an extremely powerful instrument which could, at least in theory, completely regenerate an organ like the myocardium. Some attempts in this direction have been done using embryonic stem cells in combination with collagen and extracellular matrix proteins (Matrigel®) [164] or in co-culture with optimal percentage of fibroblasts [165]. However, ES cell still presents some inconveniences such as high immunogenicity and widely documented teratomas induction after transplantation *in vivo* [132] [166] [167] [168]. Only if these problems will be overcome, the therapeutic potential of embryonic stem cells could be finally fully employed.

Some strategies to circumvent the teratoma formation might include genetic selection of differentiated ES cells [169] or ES differentiation into endothelial cells or cardiomyocytes *in vitro* before transplantation [170] [171] (e.g. it has been shown that TNF could induce the differentiation of embryonic stem cells into cardiomyocytes) [172]. However, more complications come from the unknown signalling mechanisms that might control the growth and the differentiation of ES cells; therefore it is crucial to further understand in details which are the pathways that are activated during development of these cells into differentiated tissues.

The discovery of the existence of endogenous cardiac stem cells (CSCs) into the heart which was considered until few years ago a postmitotic organ brought a highly innovative concept in regenerative medicine. The heart of mammals possesses a rare

population of stem cells which are identified by the cell-surface markers Kit [133] or Sca1 [173]. Cells expressing Kit and/or Sca1 are capable of cardiomyocytes generation *in vitro* and *in vivo* [174] and can be readily isolated on the basis of their surface markers [175]. Moreover, an additional population of cells defined by the expression of the transcription factor Isl1 has been shown to differentiate into endothelial, endocardial, smooth muscle, conduction system, right ventricular and atrial myogenic lineages during embryonic heart development [176]. Cells that express Isl1 are also present in the adult mammalian heart in the right atrium but are found in a smaller number than in the developing heart [134].

Human cardiac stem cells can be isolated and expanded after harvesting of samples of the myocardium by minimally invasive biopsy procedure [177] [178]. Therefore, autologous CSCs could be expanded *in vitro* and transplanted into patients with very small risk of immunoreaction or teratoma formation. However, due to the lack of consistent proves of CSCs effective and extensive differentiation into cardiomyocytes *in vivo*, it is difficult to define if the high CSCs self-renewal and plasticity observed *in vitro* is an artefact or not [120]. Until now there were not clinical trials utilizing these cells yet indicating that there are still several open questions that need to be answered before to move to the clinical application.

More recently, Takahashi and Yamanaka discovered that through the introduction of four defined factors (the genes: Oct3/4, Klf4, Sox2, and c-Myc) it is possible to revert a completely differentiated somatic cell such as the adult mouse tail fibroblast to an ES like cell that can undergo multilineage differentiation *in vivo* [179]. Following this result, other research groups confirmed independently these findings and the Yamanaka's team continued its work and showed the ability of these fibroblast-derived induced-pluripotent stem (iPS) cells to completely generate a new living mouse [180] [181] [182].

These cells could offer a very interesting alternative to embryonic stem cells which require a large number of donor human oocytes to be derived and for this reason raised a number of ethical and political questions [183]. Regrettably, due to the retroviral-mediated reprogramming origin of the iPS cells, viral oncogenesis (furthermore c-Myc is a well known oncogene) will greatly limit the clinical usage of these cells. In fact, it has been shown that up to 20% of the chimeric iPS mice developed tumours because of reactivation of c-Myc [180]. Nevertheless, the academic attention on the iPS cell is currently very high and iPS mediated repair of the heart after acute myocardial

infarction has been lately demonstrated by Nelson and Terzic [184]. Moreover, human iPS cells have been generated by reprogramming of differentiated human skin fibroblasts [185] [186] and alternative way to obtain human iPS have been uncovered [187]. If the undesirable tumor formation will be overcome in the future for example a patient affected by ischemic cardiomyopathy could supply his or her own skin fibroblast for derivation of "autologous" iPS cells. These cells could be proliferated *in vitro* for the generation of cardiac progenitor cells or cardiomyocytes which would be available for replacement of lost cardiomyocytes [183].

3.3. Haematopoietic stem cell mediated cardiac repair

The haematopoietic compartment of the bone marrow has been the favourite cell source for cell-transplantation-based therapies in the last seven years (clinical trials, Table 2). In the majority of these studies, the whole bone marrow mononuclear cell (BMMNC) population was utilized while in fewer trials the transplanted cells were enriched of specific subpopulation of myeloid stem cells ($CD133^+$) or myeloid progenitors ($CD34^+$). The outcomes of the clinical trials in which BMMNC were transplanted showed a general beneficial effect of the treatment and an increase in ejection fraction though with high variability within different studies [120]. The initiative to target a more defined population of haematopoietic progenitors, which is able to generate new vessels *in vivo* and has also direct or indirect cardiogenetic potential, could be the right way to further enhance the favourable effect of bone marrow derived stem cell transplantation [151].

To this end, Stamm and Steinhoff demonstrated that the intramyocardial injection of $CD133^+$ enriched BM derived cell provides beneficial effects and is promising to become a standard treatment after acute myocardial infarction [4] [5] [150] [188]. Following an experience of seven years and the achievement of phase I and phase II clinical trials they also confirmed the safety of $CD133^+$ cell isolation and transplantation [189] [190]. Other trials validated these findings demonstrating the feasibility and the benefits of $CD133^+$ cell treatment [191] [192], however, further clarification by randomized clinical Phase III trials is required [5].

At the moment, it is difficult to understand whether the selection of a specific haematopoietic subpopulation of BM derived cells is preferable to the use of the whole BMMN cells because the available data in human are still too few. On the other hand,

injection of high amount of circulating MN cells in the porcine model may induce moderate to severe hemorrhagic infarction with incidence of elevated number of $CD45^+$ cells in tissue (presumably undifferentiated haematopoietic cells or inflammatory cells) [147]. At present, it is still very complicate to decipher in details which are the correct markers or the best combination of markers that could identify the optimal haematopoietic cell subpopulation for the transplantation [151].

3.4. Mesenchymal stem cell and myocardial regeneration

As described before, mesenchymal stem cells can be easily isolated from bone marrow and expanded *in vitro* until extremely high number of cells are obtained [152]. Moreover, MSCs can be derived from several other tissues such as adipose [193], umbilical cord blood [194], peripheral blood [195], connective tissues of dermis and skeletal muscle [196].

Bone marrow derived mesenchymal stem cells (BMMSCs) have been employed to regenerate the injured myocardium with a moderate success in a number of animal models [155] [197] [198] [199]. In the clinical translation for myocardial repair, Chen *et al*. applied BMMSCs to the patients and reported significant improved left ventricular function after intracoronary application of elevated number of cells (6×10^{10}) [200]. In a successive study, the same group showed that a considerably lower amount of cells (5×10^6) did not significantly affect the ejection fraction when compared to untreated patients [120]. Beside the evaluation of cardiac function, in these trials there were neither information regarding MSCs culture conditions nor the evaluation of myocardial injuries after coronary cell infusion [199]. Indeed, while BMMNC transplantation safety has been consistently demonstrated [201], the assessments of possible adverse consequence of mesenchymal stem cells application are only recently on going [199].

Compared to BMMN cells, MSCs have to be cultured for some time *in vitro* in order to reach a certain degree of purity and an adequate number prior to transplantation. During cell manipulation (*ex vivo* expansion) microbial contamination could unfavourably affect the quality of the cell preparation or unexpected mutation may induce genetic transformation [attached manuscript 4]. In addition, the serum present in the culture medium might induce MSCs alteration with consequent host immune rejection [199]. Further troubles could appear since cultured MSCs show significantly larger dimensions compared to BMMNC (mean diameter: 20µm VS 10-

12μm, respectively) thus serious complications such as coronary embolism, pulmonary embolism and microinfarction might occur after direct intracoronary infusion [199] [attached manuscript 3].

3.5. Mechanisms of stem cell mediated cardiac repair

The stem cell therapy was born following the rousing finding of an accessible source of undifferentiated cells which could have, after direct transdifferentiation, completely rebuilt complex organs such as the myocardium. Several independent studies have verified in the animal model that stem cells from diverse sources are capable of partial functional restoration of various organs . In the specific case of the cardiac repair, it has been only lately emphasized that the degree of the restitution of functionality can not be merely awardable to the rare direct differentiation of stem cell into cardiomyocytes, smooth muscle or endothelial cells [156] [175]. Moreover, the provide evidence of new myocardium originated by transplanted stem cells has been repeatedly disputed and attributed presumably to fusion of the injected cells with resident cells other than direct differentiation [143] [144].

As an alternative explanation, it has been proposed that the observed improvements in cardiac function might be driven by paracrine mechanisms [156] [202] [203] [204]. Most likely, the mechanisms underlying cardiac reparative effects of transplanted stem cells might be both: direct and indirect. Stem cells could directly induce beneficial effects through long term engraftment and differentiation [205] and indirectly through immunomodulation and activation of host stem cells in a paracrine fashion [202] [203] [204] [206].

3.6. Challenges and future directions

The challenges to stem cell therapies for cardiac regeneration are numerous (Figure 3.2). One of the first questions that need to be answered is which type of stem cell or progenitor cell is the most suitable candidate for transplantation [8]. In order to do so, it is of primary importance to deeply understand the mechanisms by which different types of stem or progenitor cells can improve cardiac performance [9] [132]. To identify the best stem cell source, it is also necessary to test the safety of long term transplantation of each type of stem and progenitor cell in order to exclude the possibility of deleterious

side complications such as sarcoma or teratoma formation [132] [160] [166] [attached manuscript 4]. Only after accurate investigation it will be possible to achieve more rational cell-based approaches for cardiac diseases and perhaps target the best stem cell candidate for a specific cardiac pathology (e.g. acute myocardial infarction and chronic ischemic cardiomyopathy could require different types of stem or progenitor cell) [120].

An additional issue that need to be addressed for stem cell therapies is defining the most favourable administration route [attached manuscript 3]. Stem cells have been injected intravenously, into coronary arteries or directly into the myocardium [120]. Reports of numerous phase I trials revealed the occurrence of few serious adverse effects [149]; however, the capacity of cells to remain in the area of injury is highly dependent on the delivery strategy [8] [138] [attached manuscript 3].

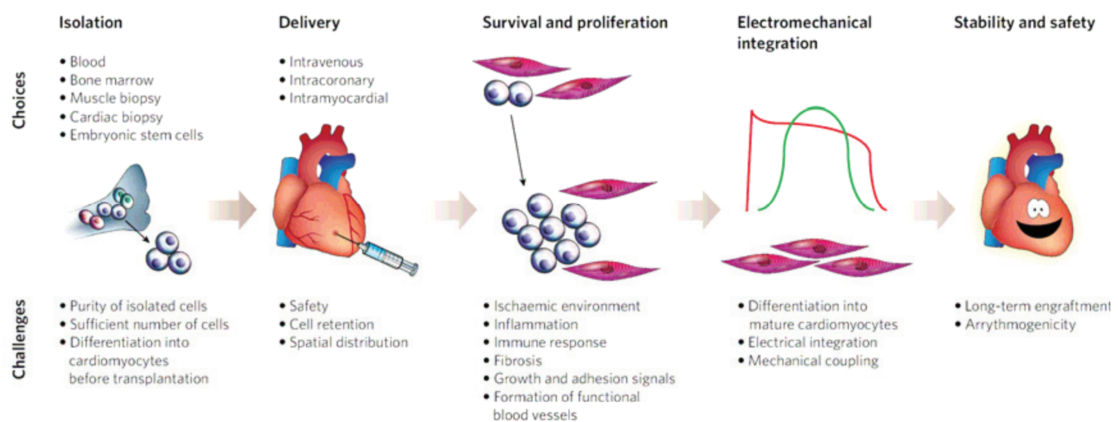


Figure 3.2. Challenges to stem cell therapies for cardiac diseases. The effective cardiac regeneration with stem cell therapy will entail cautious consideration at each step from cell isolation to their stable and safe long term engraftment [120].

Finally, another challenge that needs to be faced is the survival of the cells in the inflammatory environment of the infarcted myocardium, indeed generally up to 90% of the transplanted cells die within a week [132].

Stem cell based therapies for cardiac repair will need the identification of appropriate structural and functional properties for cell graft and the improvement of survival and long term electromechanical stability / integration of the cells into the damaged tissue [207]. Survival and integration of the cell after transplantation could be enhanced by the usage of matrices like collagen [138] or Matrigel ® [170]. The process could be further

ameliorated with the concomitant induction of revascularization and simultaneous nanofibers mediated growth factor release [208]. Another direction could include the identification of the stem cell-derived paracrine factors which are responsible for the beneficial effect of stem cell transplantation and their employment as therapeutically agents. The direct administration of cytokines or growth factors could be much more controllable and reproducible than transplantation of heterogeneous population of stem or progenitor cells.

4. Drugs driven stem cell mobilization

4.1. Granulocyte colony-stimulating factor

The recognition of different cell types with cardiogenic potential has raised attention in restoring cardiac function by the activation and mobilization of endogenous stem and progenitor cells without the need of *ex vivo* cell manipulation. Proangiogenic factors released by ischemic tissues or applied exogenously could mobilize EPCs to the area of injury and improve blood perfusion [9] [175].

In addition to the mobilization stimulus, which recruits stem cells from the bone marrow to the peripheral blood, homing signals might be crucial to lead the cells to the infarcted myocardium. It has been shown that the chemoattractant cytokine stromal derived factor 1 (SDF-1α) can induce homing of EPCs to the heart [209] and can influence EPCs rolling and adhesion capacity on the microvascular endothelium (rolling and adhesion is considered to be the first stage of extravasation) [210]. Furthermore, not only haematopoietic stem or progenitor cells are circulating in the peripheral blood but also mesenchymal stem cells can be found there although in a very limited number [211]. This finding suggested that non-haematopoietic stem or progenitor cells might have the ability to home to damaged tissues.

Moreover, other stem cells like CSCs reside in clusters (niches) in the heart in a silent status and could be activated by specific factors [177]. These factors that could stimulate CSCs to leave their putative niches and move to an injured area have not yet been identified. However, other cytokines or growth factors that are capable of haematopoietic stem and progenitor cell mobilization have been already defined and some of them e.g. the granulocyte colony stimulating factor (G-CSF) are in use since years in the clinical setting [212] [213] [214] [215] [216].

G-CSF induces the proliferation and differentiation of hematopoietic progenitor cells and the release of mature granulocytes from bone marrow. It also triggers the mobilization of $CD34^+$ haematopoietic stem cells in circulating blood [212] [214]. Due to its features G-CSF has been utilized successfully in various preclinical investigations as agent to counteract myocardial damage after myocardial infarction [217] [218] [219]. Following these encouraging outcomes in the animal model, some clinical trials have also been performed demonstrating that G-CSF treatment is safe but does not seem to induce significant improvement in ventricular function after acute MI in unselected

patients [220] [221]. However, meta-analysis of randomized controlled trials suggested that G-CSF treatment might be beneficial when started early and when administrated to acute MI patients with LV dysfunction [221].

The interest in recognizing the pivotal molecules which are able to activate the host stem and progenitor cell is rising constantly. The identification of such factor or combination of factors could ease the complications which are now linked to stem cell therapies like e.g. the individuation of the ideal stem cell type, *ex vivo* manipulation of the cells, long term side effects of transplantation and immunoreaction.

4.2. Erythropoietin

Erythropoietin (EPO) is a hypoxia-induced glycoprotein hormone that stimulates the proliferation and differentiation of erythroid precursor cells in order to counteract diminished oxygen levels, including those caused by anaemia and hypoxia [222] [223]. It can inhibit hypoxia-induced apoptosis of cardiomyocytes and protect cardiomyoblasts from hydrogen peroxide-induced injury [224] [225] [226]. EPO can also induce neo-vascularization [227], reduce the infarction size and confer effective cardiac protection against ischemia-reperfusion injury and chronic heart failure [228] [229] [230]. This combined evidence has fuelled significant interest in the potential use of EPO as a cytoprotective agent in the cardiovascular system.

Numerous studies revealed that the transplantation of endothelial progenitor cells (EPCs) isolated from bone marrow (BM) and peripheral blood results in neovascularization of the ischemic tissues [231] [232] [233] [234]. Current findings demonstrate EPO treatment can mobilize EPCs in animals and humans [235] [236] [237] and increase the adhesive and proliferative properties of circulating EPCs [238]. However, it is still not clear whether EPO-mobilized EPCs can migrate into the ischemic myocardium and participate in the process of myocardial regeneration.

One of the aims of this thesis was to evaluate the therapeutic efficacy of intracardiac EPO injection in the infarcted heart. We assessed if intracardiac injection of EPO could recruit stem and progenitor cells to the infarcted heart by activating stem cell homing signalling to promote cardiac regeneration after myocardial infarction [attached manuscript 1].

Our findings demonstrated that a single intracardiac injection of EPO (3000U/kg) after MI significantly up-regulated stem cell homing factor SDF-1α and increased the

number of c-Kit^+ and $CD34^+$ stem cells initially (24h) in the peripheral blood and subsequently (48h) in the myocardium (Figure 4.1). Moreover, the local administration of EPO: reduced infarction size, inhibited cardiac remodeling (decrease of: myocardium loss, cardiomyocyte compensatory growth and interstitial fibrosis), enhanced cardiomyocyte protection (reduced apoptosis and up-regulation of pro-survival factor Bcl2 mRNA) and improved cardiac function (Table 4.1 and 4.2) [attached manuscript 1 and 2]. We also found reduced cardiac troponin T (cTnT) plasma levels and improved right ventricle (RV) loading conditions in EPO treated animals, which may indicate a better quality of life and reduced long-term mortality secondary to heart failure [239] [240].

The local administration of EPO described in the attached manuscripts 1 and 2 might achieve the same high local concentration reached by a prolonged systemic approach but with the reduction of complications associated with prolonged systemic administration. Recently, two independent studies indicated that the cardioprotection associated with EPO treatment might be dose-dependent, with higher doses of Darbepoetin-α, an EPO analogue, producing better protection than lower doses against anterior wall thinning, LV dilatation, and LV systolic dysfunction [241] [242]. However, prolonged EPO exposure is likely to cause significant adverse effects on haematocrit and blood flow properties, resulting in vascular thrombosis and even excess mortality [243] [244]. In this study, EPO treatment was accomplished with a single intracardiac injection, which did not lead to intramural thrombus formation or abnormal effects on haematocrit. Moreover, circulating EPO levels were traced and side effects on haematocrit and thrombus formation in other organs have not been observed. We suggest that a single, intracardiac administration of EPO may represent an alternative to systemic delivery, reducing the dose required for effective cardioprotection and minimizing side effects by limiting the potential for systemic toxicity and vascular thrombosis.

The underlying mechanism by which intracardiac injection of EPO improves cardiac function after MI has not been clearly identified, but our results highlighted several beneficial dynamics acting in concert: (1) preservation of viable myocardium from ischemia (indicated by reduction of infarction size) and revascularization. (2) Mobilization of CD34+ and c-Kit+ stem/progenitor cells in peripheral blood at 24 hours. (3) SDF-1α up-regulation which induced early recruitment of stem/progenitor cells to the infarcted heart at 48 hours (Figure 4.2). (4) Anti-inflammatory and

cytoprotective direct effects of EPO. Most likely, EPO mediated directly: cytoprotection and angiogenesis and indirectly: (through stem/progenitor cells activation, mobilization and homing) partial regeneration of the myocardium and a redundant beneficial effect enhanced by stem/progenitor cell paracrine action (further cytoprotection and angiogenesis) [attached manuscripts 1 and 2].

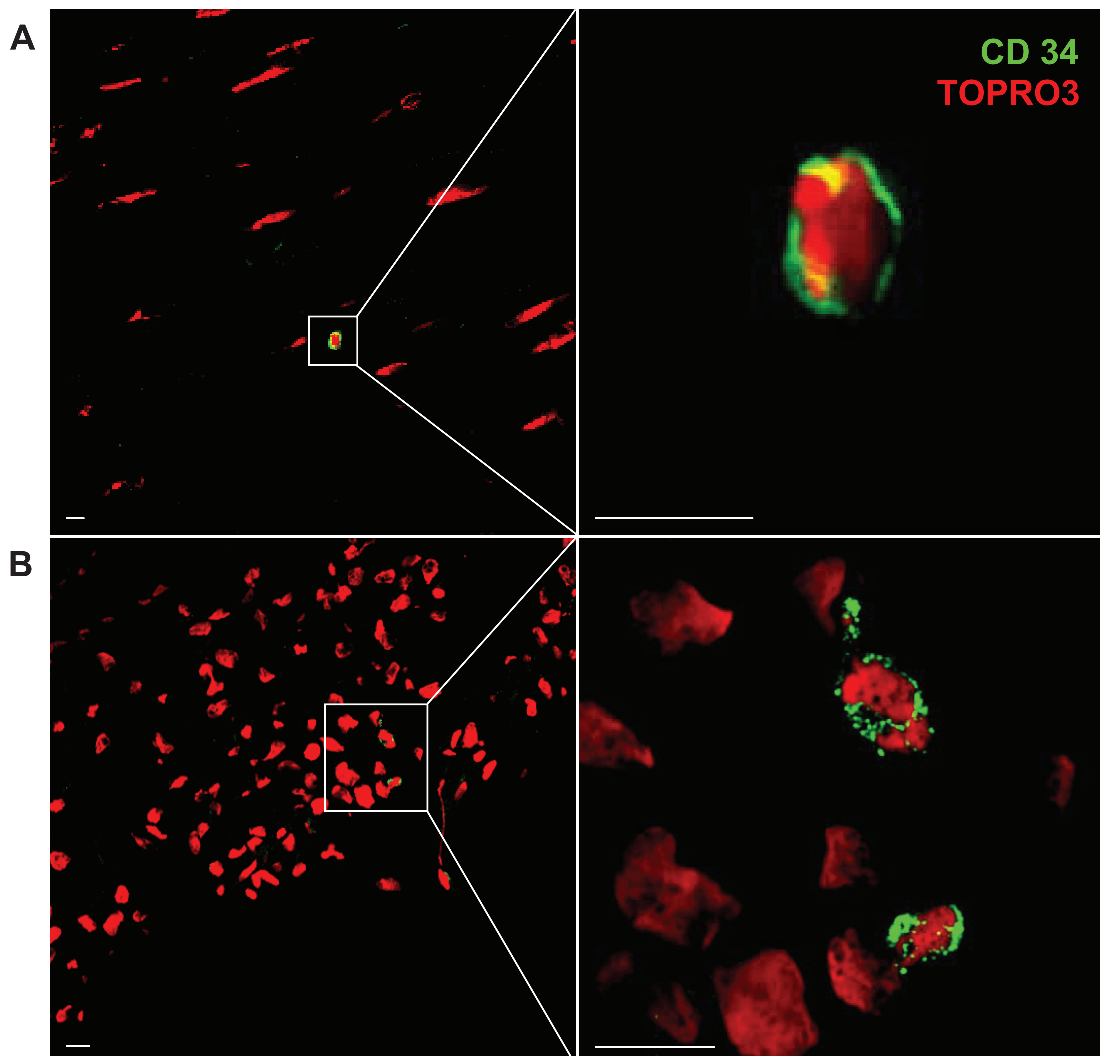


Figure 4.1. Representative images for CD34$^+$ (green) cells (square) in NIZ (A, Bar = 5 µm) and IZ (B, Bar = 10 µm) at 48 hrs after EPO treatment. Red, TOPRO3 in nuclei [attached manuscript 1].

Despite our encouraging findings, the exact effects of EPO on the endogenous cardiac stem cells (CSCs) are still not clear. EPO has been shown to regulate the proliferation and differentiation of embryonic and adult neural stem cells *in vitro* and *in*

vivo [245]. As previously mentioned, it is known that adult CSCs, negative for the expression of blood lineage markers (Lin-) but positive for stem cell marker c-Kit, are multipotent and support myocardial regeneration [133]. Hence, it can be speculated that EPO delivered by intracardiac injection may also mediate CSCs proliferation and differentiation to regenerate the infarcted myocardium. Further studies need to be conducted in order to address this hypothesis.

The molecular mechanisms associated with EPO-mediated cardiac protection have not been satisfactorily elucidated. In this study, we observed increase in the mRNA levels of pro-survival signal Akt and its downstream target eNOS at 24 and 48 hours, which might be closely associated with cardioprotective effects of EPO. Our findings are consistent with previous reports that indicated EPO protects cardiomyocytes from apoptosis via up-regulation of eNOS and activation of Akt [246] [247] and that eNOS is required for SDF-1α-mediated c-Kit$^+$ HSCs directional migration [210].

As previously written, myocardial necrosis progresses within 2-6 hours after the onset of MI [16] [18] and prompt reperfusion within this narrow time window significantly decreases early mortality [248]. Administration of EPO during the therapeutic window significantly reduces infarction size and improves cardiac function [224] [225] [226]. Hence, local injection of EPO during emergency coronary artery bypass graft surgery after acute MI could be an optimal approach for EPO treatment.

This study suggests that the beneficial effects of EPO treatment might be closely associated with the targeted migration of stem/progenitor cells. Moreover, EPO therapy is effective and feasible when delivered directly into the myocardium using a clinically relevant approach. The results reported herein establish EPO as a stem cell modulating hormone that facilitates cardiac regeneration. The effective pharmacological agents (such as EPO) that may be applied during coronary interventions or cardiac surgery to promote early cardiac preservation and stem cell activation are highly desirable. Accordingly our present findings have obvious translational implications for the treatment of patients with acute coronary syndrome. Further development of the concept will reveal whether these encouraging animal data can be translated into clinical applications [attached manuscripts 1 and 2].

Parameter	Sham (n=11)	MIC (n=14)	MI-EPO (n=11)	*P**
Pmax (mmHg)	147.74 ± 3.36	114.95 ± 6.94	126.70 ± 7.12	0.255
dp/dt max (mmHg/s)	10942.50 ± 276.37	5815.23 ± 335.97	7374.84 ± 525.45	0.016
-dp/dt max (mmHg/s)	-10137.44 ± 281.57	-3453.68 ± 121.38	-4743.93 ± 480.98	0.007
Relaxation time (msec)	8.05 ± 1.53	19.74 ± 1.40	13.07 ± 1.37	0.003
EDV (µl)	211.30 ± 14.62	312.44 ± 27.98	313.56 ± 23.52	0.784
ESV (µl)	103.24 ± 5.96	225.36 ± 20.04	187.49 ± 15.60	0.101
SV (µl)	108.06 ± 10.31	87.11 ± 11.52	126.07 ± 12.18	0.031
EF (%)	50.45 ± 2.08	27.12 ± 1.78	40.05 ± 2.42	<0.001
SW (µlxmmHg)	13124.94 ± 1694.95	6166.67 ± 846.91	11742.17 ± 1352.87	0.001
HR (1/min)	418.16 ± 10.19	359.61 ± 19.73	409.09 ± 11.32	0.055

Table 4.1. Hemodynamic of the LV under Baseline Conditions 6 weeks after MI

Values are represented as Mean ± SEM, * MIC vs. MI-EPO, Pmax means maximum pressure; dp/dt indicates peak rate of maximum pressure rise (dp/dt max) and decline (-dp/dt max); EDV, end-diastolic volume; ESV, end-systolic volume; SV, stroke volume; EF, ejection fraction; SW, stroke work; and HR, heart rate.

Parameter	Sham (n=11)	MIC (n=14)	MI-EPO (n=11)	*P**
Pmax (mmHg)	144.54 ± 3.74	124.08 ± 3.34	131.05 ± 4.37	0.209
dp/dt max (mmHg/s)	18962.22 ± 358.66	9529.60 ± 490.22	12456.81 ± 726.55	0.002
-dp/dt max (mmHg/s)	-9418.62 ± 349.47	-5421.46 ± 355.71	-6741.31 ± 538.93	0.045
Relaxation time (msec)	5.95 ± 1.04	13.76 ± 1,51	8.92 ± 1.13	0.022
EDV (µl)	186.24 ± 14.14	299.74 ± 30.16	315.01 ± 16.06	0.684
ESV (µl)	48.08 ± 5.23	203.67 ± 21.44	160.20 ± 16.02	0.136
SV (µl)	138.06 ± 10.06	96.13 ± 13.37	154.77 ± 20.15	0.019
EF (%)	74.44 ± 1.54	31.61 ± 2.25	48.39 ± 5.01	0.003
SW (µlxmmHg)	16336.62 ± 1334.16	7428.62 ± 1050.69	14937.78 ± 1971.47	0.004
HR (1/min)	474.30 ± 10.06	429.92 ± 8.29	459.30 ± 7.85	0.019

Table 4.2. Hemodynamic of the LV under Stress Conditions 6 weeks after MI

Values are represented as Mean ± SEM, * MIC vs. MI-EPO, Pmax means maximum pressure; dp/dt indicates peak rate of maximum pressure rise (dp/dt max) and decline (-dp/dt max); EDV, enddiastolic volume; ESV, endsystolic volume; SV, stroke volume; EF, ejection fraction; SW, stroke work; and HR, heart rate.

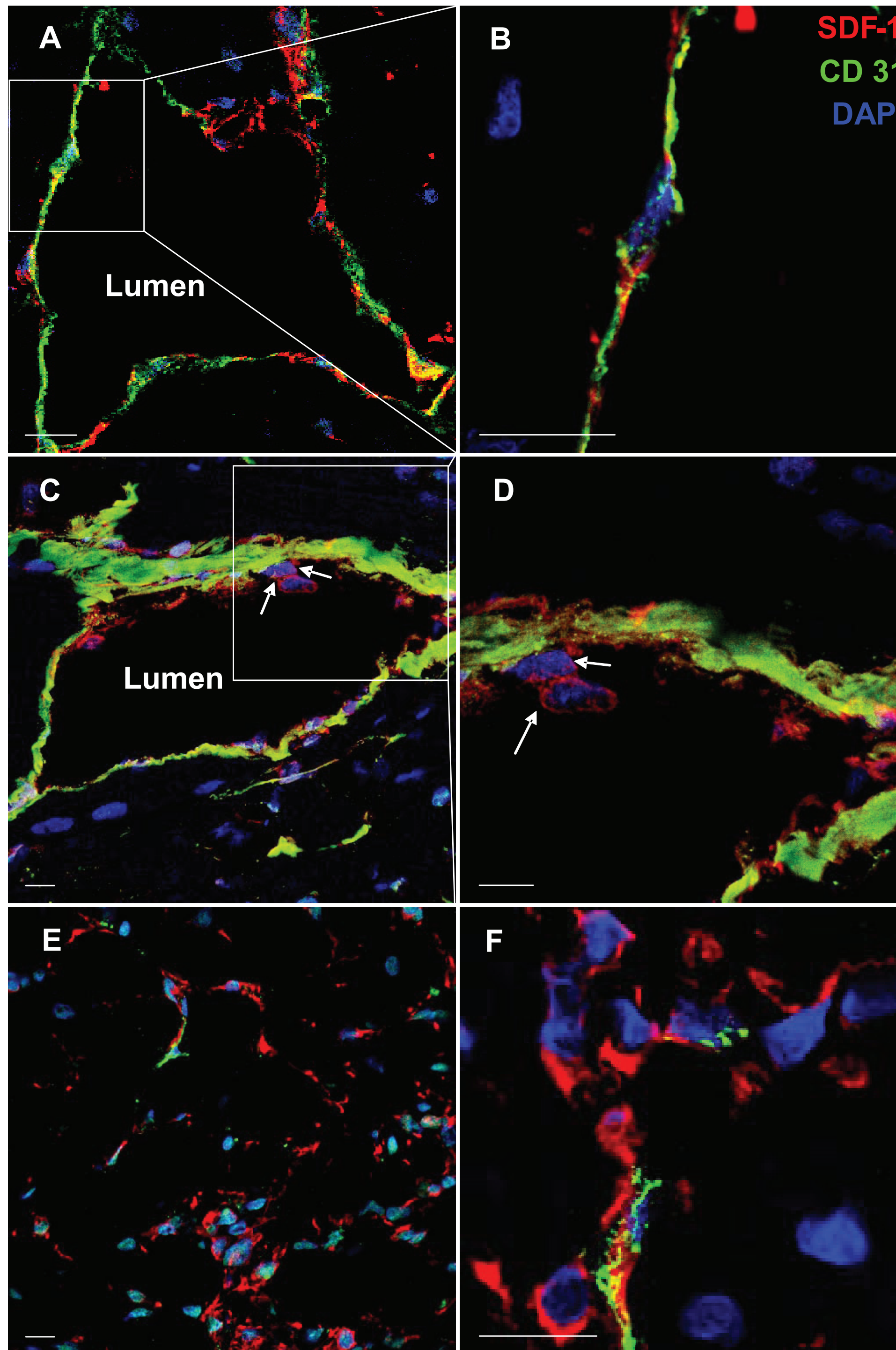


Figure 4.2. (A-D) EPO injection up-regulated SDF-1α. In NIZ of MI-EPO, most SDF-1α + cells (red) co-localized with CD31 (green). Occasionally, cell adhesion (arrows in C and D) with the SDF-1α + endothelial cells was visible. (E, F) In contrast, a number of SDF-1α^{+} cells (red) in IZ of MI-EPO hearts did not co-localize with CD31 (green). Scale bars = 10 µm. Blue, DAPI in nuclei [attached manuscript 1].

5. Molecular mechanisms of stem cell mobilization and homing

5.1. SDF-1α mediated mechanisms of stem cell mobilization

The crucial role of chemokines in stem cell trafficking has been intensively investigated during the last years and the central function played by SDF-1α/CXCR4 axis has been broadly highlighted [210] [249]. SDF-1α is a survival and proliferation factor for haematopoietic stem cells [250] [251]. It is produced in many organs, including the bone marrow where is expressed by stromal cells (e.g. osteoblasts) [252]. SDF-1α is a powerful chemoattractant for immature and mature hematopoietic cells of several lineages [253] [254] [255] [256]. Its receptor CXCR4 is expressed on lymphocytes, myeloid cells, megakaryocytes and HSCs and facilitates these cells to migrate across a gradient of SDF-1α concentrations [257] [258] [259].

In normal conditions, bone marrow stromal cells establish a local SDF-1α concentration gradient that is the primary signal for stem cells (SCs) homing [260]. During the mobilization of stem cell, the level of SDF-1α decreases in the BM while the expression of CXCR4 rises creating a gradient of chemoattraction to the peripheral blood [257]. The decrease of SDF-1α level in the BM is primarily due to its degradation by induced proteases such as cathepsin G, matrix metalloproteinases and neutrophil elastase [261] [262] [263] [264]. These proteases also trigger the degradation of central adhesion molecules such as very late antigen-4 (VLA-4, an integrin dimer composed of CD49d, integrin-α, and CD29, integrin-β) and its cognate receptor: vascular cell adhesion molecule-1 (VCAM-1) [249] [265]. Altogether: SDF-1α gradient reversion, CXCR4 upregulation and adhesion molecules degradation initiate the release of stem cells from the bone marrow to the peripheral blood [249].

An additional mechanism for SCs mobilization mediated by direct SDF-1α stimulation has been proposed lately by our research group. We showed that SDF-1α treatment *in vitro* directly induce the modification of F-actin cytoskeleton and the distribution of CXCR4 receptor in c-kit^{+} cell [210]. In our *in vitro* model, SDF-1α stimulation provoked the polarization of CXCR4 surface marker at the filopodia and a rearrangement of the actin cytoskeleton of c-kit^{+} cells. The redistribution of CXCR4 and the formation of membrane protrusions were consistent with the *in vitro* cell migration assay (Boyden chamber) (Figure 5.1 and 5.2) [210].

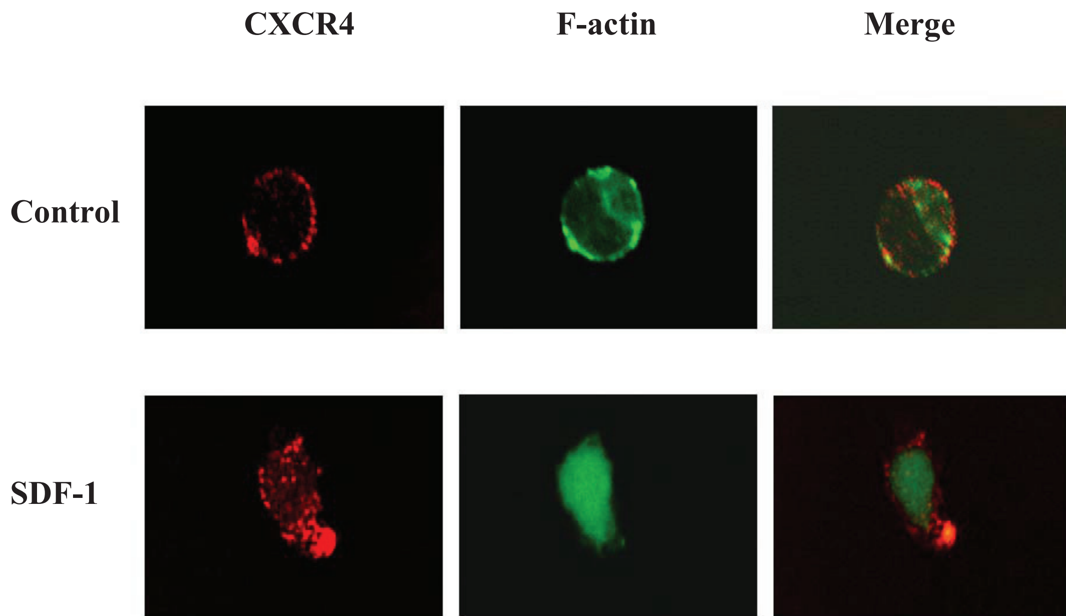


Figure 5.1. Confocal laser microscopy of F-actin and CXCR4 protein distribution in c-kit$^+$ cells after SDF-1α treatment *in vitro*. c-kit$^+$ cells were extracted after migration in the Boyden chamber assay with and without SDF-1α stimulation. In cells without SDF-1α stimulation, CXCR4 (red fluorescence) and F-actin (green fluorescence) expression was equally distributed along the plasma membrane. After SDF-1α treatment, a reorganization of the cytoskeleton represented by a modified intracellular F-actin distribution and a concentration of CXCR4 protein at the filopodia have been detected (magnification: ×1000).

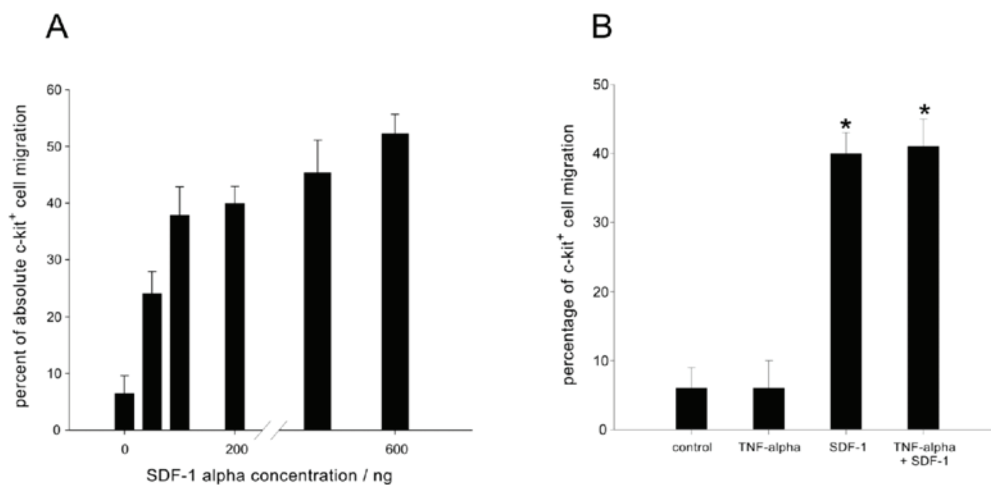


Figure 5.2. Quantitative analysis of c-kit$^+$ cell migration in Boyden chamber assays. Data are expressed as mean ± SD (*P<0.05 vs control).

5.2. SDF-1α and EPO mediated mechanisms of SCs transmigration

The initial phases of transmigration of stem cells in the surrounding tissues are characterized by the interactions of circulating cells with the vascular endothelium. These processes are defined as rolling and adhesion and are regulated by growth factors, chemokines and cytokines released in the extracellular milieu as well as receptors expressed in both stem cells and endothelial cells [210].

It has been shown that MSCs interaction with the endothelium occurs through P-selectin-mediated rolling and VCAM-1/VLA-1-mediated firm adhesion [266]. Moreover, the firm adhesion of leukocyte in the cremaster muscle induced by TNF-α seems to be mediated by an upregulation of local intercellular adhesion molecule-1 (ICAM-1) [267] [268]. Regarding HSCs ($CD34^+$) mobilization processes, several studies have been reported on the involvement of adhesion molecules such as CD49d (VLA-4); CD49e (VLA-5) and its cognate receptor, vascular adhesion molecule-1 (VCAM-1) [269] [270] [271] [272]; LFA-1 (CD18/CD11a) and its cognate receptor, intracellular adhesion molecule-1 (ICAM-1); and CD62L (L-selectin) and its cognate receptor on endothelial cells [273] [274] [275] [276].

Our group provided novel information about the mechanisms that regulate SDF-1α and EPO mediated homing of SCs. We demonstrated that SDF-1α induced firm adhesion of HSCs is abolished by desensitization of SDF-1α/CXCR4 signalling pathway and by blockage of ICAM-1 on the microvascular endothelium [210]. Additionally, we found that cytokines and growth factor such as SDF-1α and EPO are capable to directly stimulate the upregulation of pivotal adhesion molecules (e.g. ICAM-1, VCAM-1, P-selectin) in endothelial cells *in vitro* (Figure 5.3) [data not published]. Thus in accordance with other studies, endogenous cytokines or exogenously administered growth factors and cytokines could facilitate the process of engraftment directly acting on adhesion molecules regulation [249] [267] [268] [277].

In our SDF-1α study we highlighted a new mechanism on peripheral stem cell–endothelium interactions. We discovered that eNOS is required for SDF-1α mediated adhesion of SCs on the microvascular endothelium [210]. Furthermore, the inhibition of nitric oxide synthases (NOS) enzymes by L-NAME in presence of SDF-1α stimuli decreased the adhesion of c-kit^+ cell inducing major adhesion of endogenous leukocytes [210].

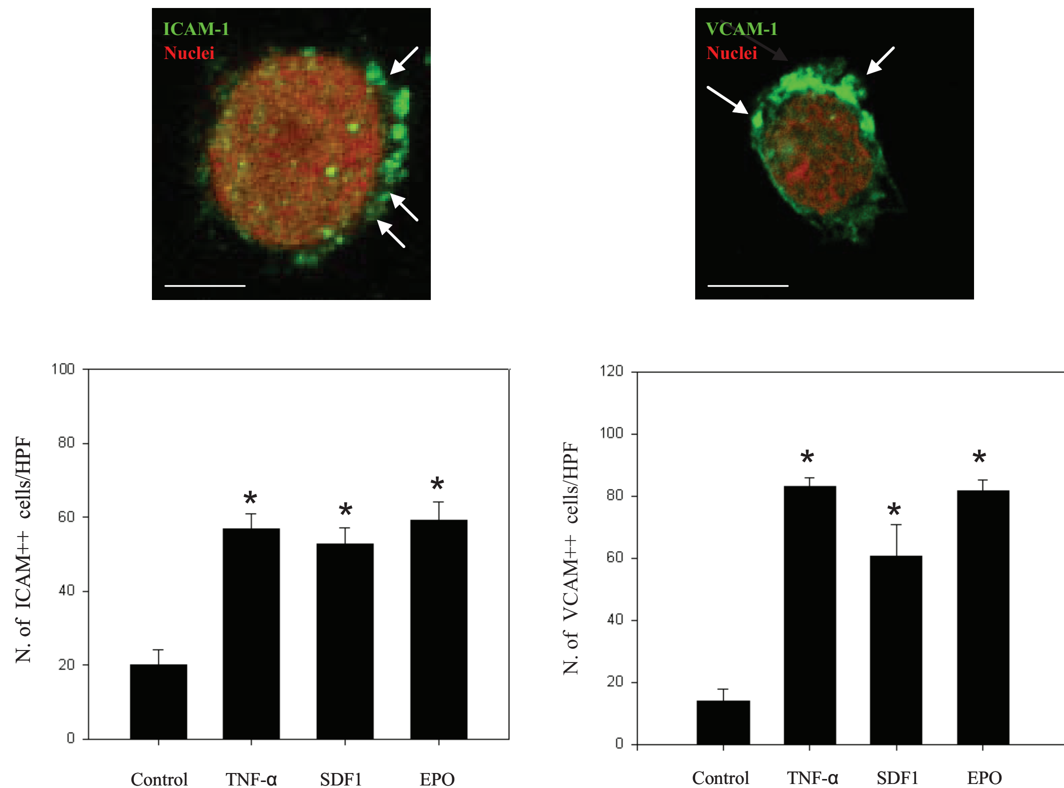


Figure 5.3. Representative confocal microscope images of adhesion molecules expression. Scale bars = 40 µm. Red, TO-PRO3 in nuclei. Quantitative analysis of SVEC cell overexpression of ICAM-1 and VCAM-1, after *in vitro* stimulation with SDF-1α and EPO. TNF-α is used as positive control. Data are expressed as mean ± SD (*$P<0.05$ vs control).

5.3. eNOS and EPO mediated mechanisms of SCs mobilization and homing

On the other hand, the essential role of eNOS for SCs mobilization has been already underlined in bone marrow stromal cell. The nitric oxide (NO)-mediated signalling pathways have been shown to be necessary for SCs mobilization [278] [279] [280] [281]. Aicher *et al.* [279] [282] were able to demonstrate that endothelial nitric oxide synthase (eNOS) is activated in bone marrow stroma by a proximal stimulus (in this case VEGF); downstream, NO then undergoes S-nitrosylation by paracrine mechanisms and triggers MMP-9, which releases a stem cell–active cytokine, soluble Kit ligand (sKitL) (Figure 5.4) [278]. sKitL leads endothelial progenitor and hematopoietic stem cells to switch from a quiescent to a proliferative niche and induces fast stem cell mobilization to the peripheral blood [279] [282].

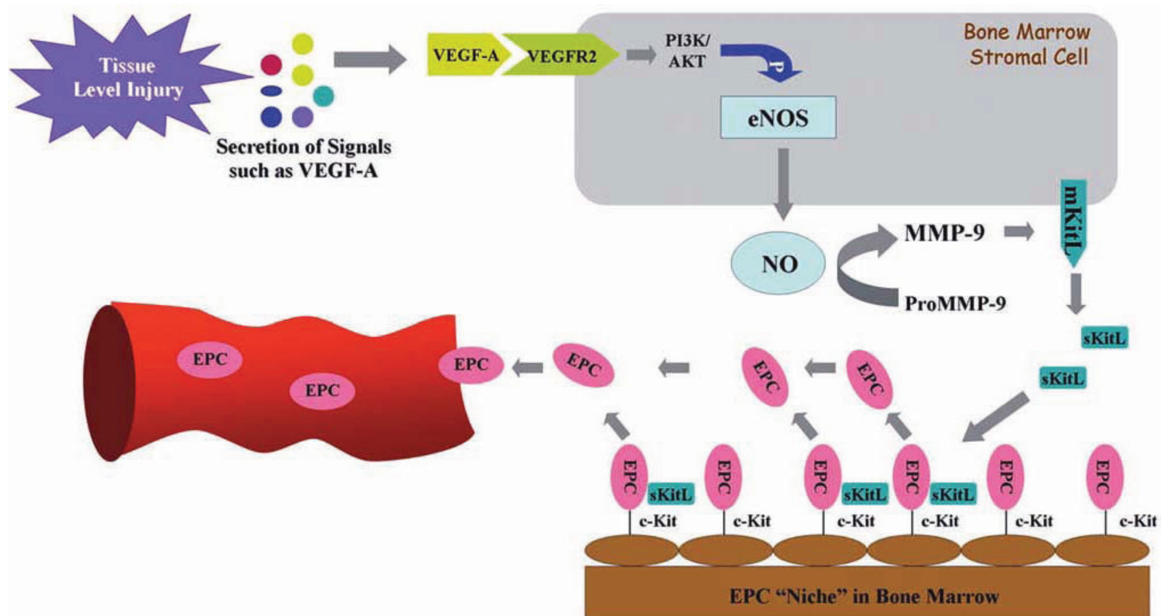


Figure 5.4. eNOS–NO–MMP-9–KitL. Signal molecules, such as VEGF, are released by injured tissue. VEGF and other mobilizing stimuli might activate the eNOS pathway through their cognate receptors signaling [278].

In our EPO study [attached manuscript 1] we emphasized that EPO is capable to activate Akt and eNOS in the heart and to increase SCs number at first in peripheral blood and secondarily into the myocardium through SDF-1α upregulation. After binding its receptor (EPOR), EPO is well known to activate several signalling pathways including the P13K/Akt [283] [284]. Possibly, EPO might also act as a proximal stimulus for bone marrow stromal cell and after binding EPOR activate a pathway similar to the one proposed by Aicher *et al.* [279] [282]. On the other hand, EPO could induce additional secreted signals (e.g. VEGF) for BM stromal cells and lead to haematopoietic stem cell release indirectly (Figure 5.5). Further investigation is mandatory to uncover the details of erythropoietin mediated stem cell mobilization and homing [285].

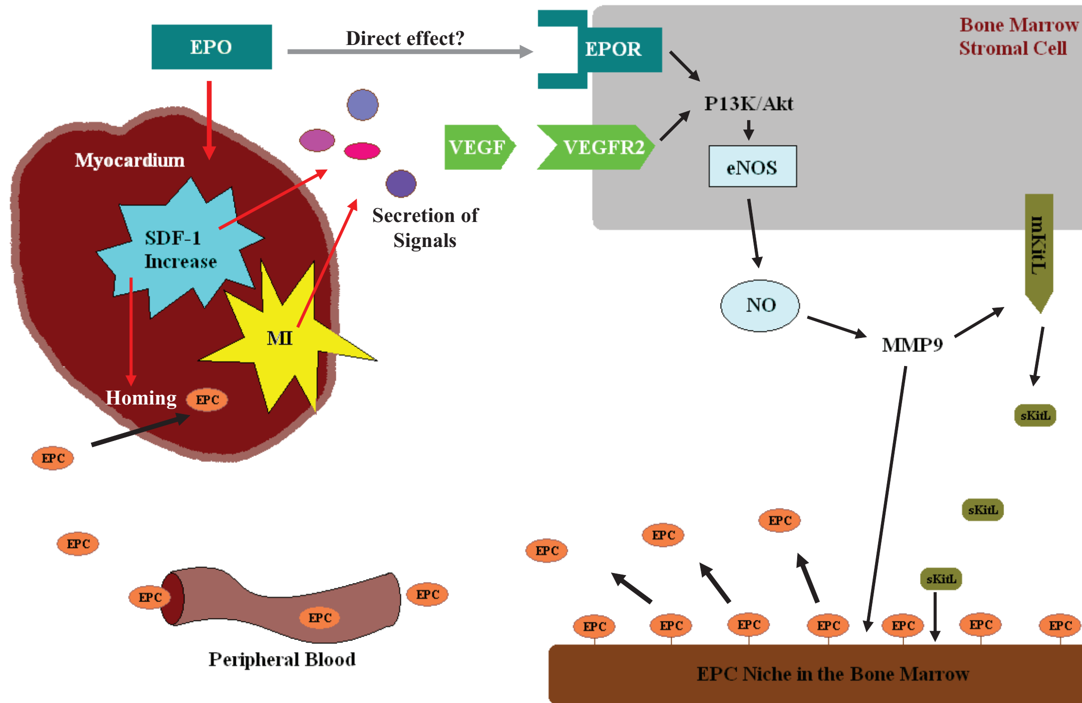


Figure 5.5. Our proposed mechanism of stem cell mobilization enhanced by EPO.

5.4. SDF-1α and CXCR4 axis in the heart

Our study on the effects of erythropoietin administration to the infarcted myocardium uncovered the early kinetics of SDF-1α/CXCR4 *in vivo* [manuscript 1]. The mRNA levels of SDF-1α/CXCR4 were investigated in the infarcted area of the myocardium as well as in the remote zone. As expected, the results showed a discrepancy in the expression levels of SDF-1α and its receptor within the two areas (IZ and NIZ) probably due to the highly different distribution of cell types [manuscript 1]. In fact as discussed in chapter 2, the adverse cardiac remodelling that immediately follows MI creates an alteration in the natural ratio of cardiomyocytes, endothelial cells and myofibroblasts.

In order to further clarify the expression patterns of SDF-1α and CXCR4 in the specific cell types of the myocardium, we investigated the effects of erythropoietin stimulation on cardiomyoblasts and endothelial cells *in vitro* (mRNA levels by real time PCR) [data not published]. Interestingly, both cell types showed high upregulation of SDF-1α after EPO stimulus but in the endothelial cell the response was faster (Figure

5.6). The hypoxia stimulus enhanced the velocity of cell reaction in the cardiomyoblast, while endothelial cell showed a kinetic similar to the one of normoxia (Figure 5.7). Remarkably, CXCR4 mRNA level was found to be strongly downregulated in concomitance with SDF-1α upregulation (in normoxia conditions) suggesting the presence of a possible feedback regulatory mechanism (data not shown). This finding is consistent with the study of Peled *et al*. where high concentration of SDF-1α have been shown to downregulate the expression of CXCR4 *in vitro* and *in vivo* [286].

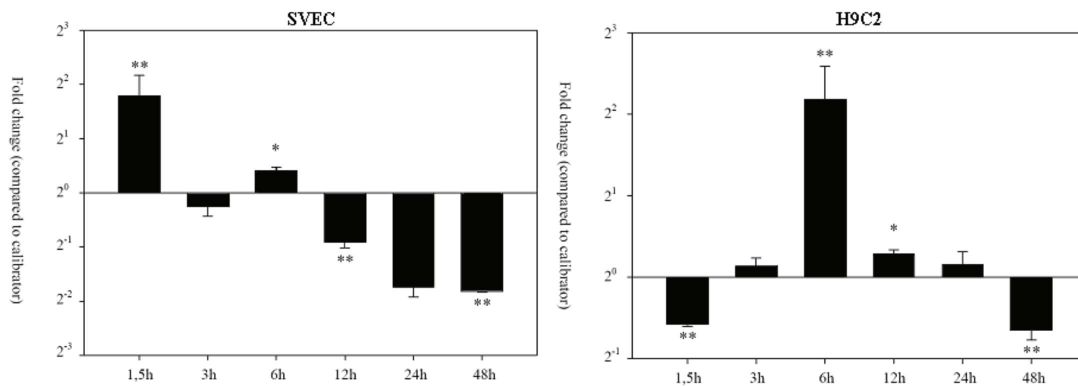


Figure 5.6. SDF-1α expression levels in SVEC and H9C2 cell after EPO stimulation *in vitro*. Data are expressed as mean ± SEM (*P<0.05 vs calibrator; **P<0.01 vs calibrator). Calibrator = Line.

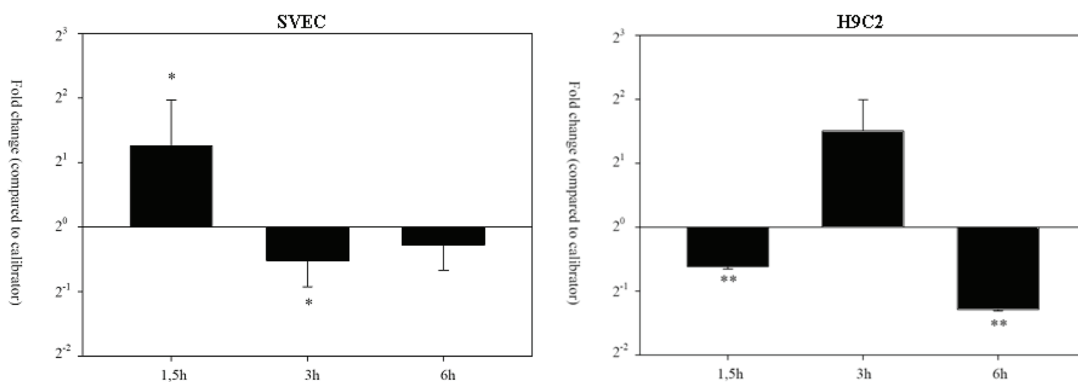


Figure 5.7. SDF-1α expression levels in SVEC and H9C2 cell after EPO stimulation *in vitro* under hypoxia conditions. Data are expressed as mean ± SEM (*P<0.05 vs calibrator; **P<0.01 vs calibrator). Calibrator = Line.

With these data we confirmed the direct action of erythropoietin on two predominant cell types of the myocardium. EPO stimulation *in vitro* significantly modulated SDF-1α/CXCR4 expression in cardiomyoblast and endothelial cell both under normoxia and hypoxia conditions. These findings suggest that in the myocardium EPO regulates SDF-1α/CXCR4 axis not only by a specific cell type but through the major cell components of the heart. Most likely, the modulation of SDF-1α/CXCR4 axis mediated by EPO takes place with the synergistic activity of other cardiac cell components such as myofibroblasts and stem cells or infiltrating inflammatory cells.

6. Stem cell administration route

6.1. Intraarterial application

As described in chapter 3, numerous delivery approaches exist for cell-based therapy. In order to achieve the optimal regeneration potential, the desired routes of stem cells delivery should be chosen to be tailed to the character of individual stem cell population, its targeted tissues and its therapeutic purpose. Intraarterial injection of stem cells as one of the commonly executed routes has been used in several preclinical settings [287] [288] [289]. As being one of the most attractive fields for stem cell based therapies, different types of cells as well as MSCs were also used for myocardial regeneration including intracoronary transplantation route [290] [291]. However, only few studies underlined the kinetics of MSCs after intaartery administration [288] [290] [291].

Considering that MSCs size might be much larger than the capillaries size, additional aim of this thesis was to investigate the immediate term behaviours of human adipose derived MSCs when are injected intraarterially in a small animal model of intravital microscopy. We hypothesized that cultured MSCs might be relatively large cells which may not be suitable for intravascular transplantation [attached manuscript 3].

Expanded human MSCs population exhibited typical markers, positive for CD29, CD44, CD73, CD90, and CD105 [data supplement, attached manuscript 3]. The average cell size is ranged between 16 µm and 53 µm in suspension (median=30.5±8.6, n=117) and between 20.1 µm and 95.9 µm in adhesion (median=47.2±16.7, n=108) (Figure 6.1), which is in agree with previous report Vulliet *et al*. [291]. However, the diameter of human post-capillary venules range approximately between 10-50 µm and is even smaller in arterioles (8-30 µm) [292]. Moreover, it is proposed that capillaries size average 8 µm in diameter [292] [293] [294] [295] [296], a dimension definitely smaller than MSCs regular diameter. MSCs have shown to be in general larger in dimension than capillaries and precapillary of the mouse cremaster when compared in vivo with precapillary vessel (both arterial and vein) diameter [attached manuscript 3].

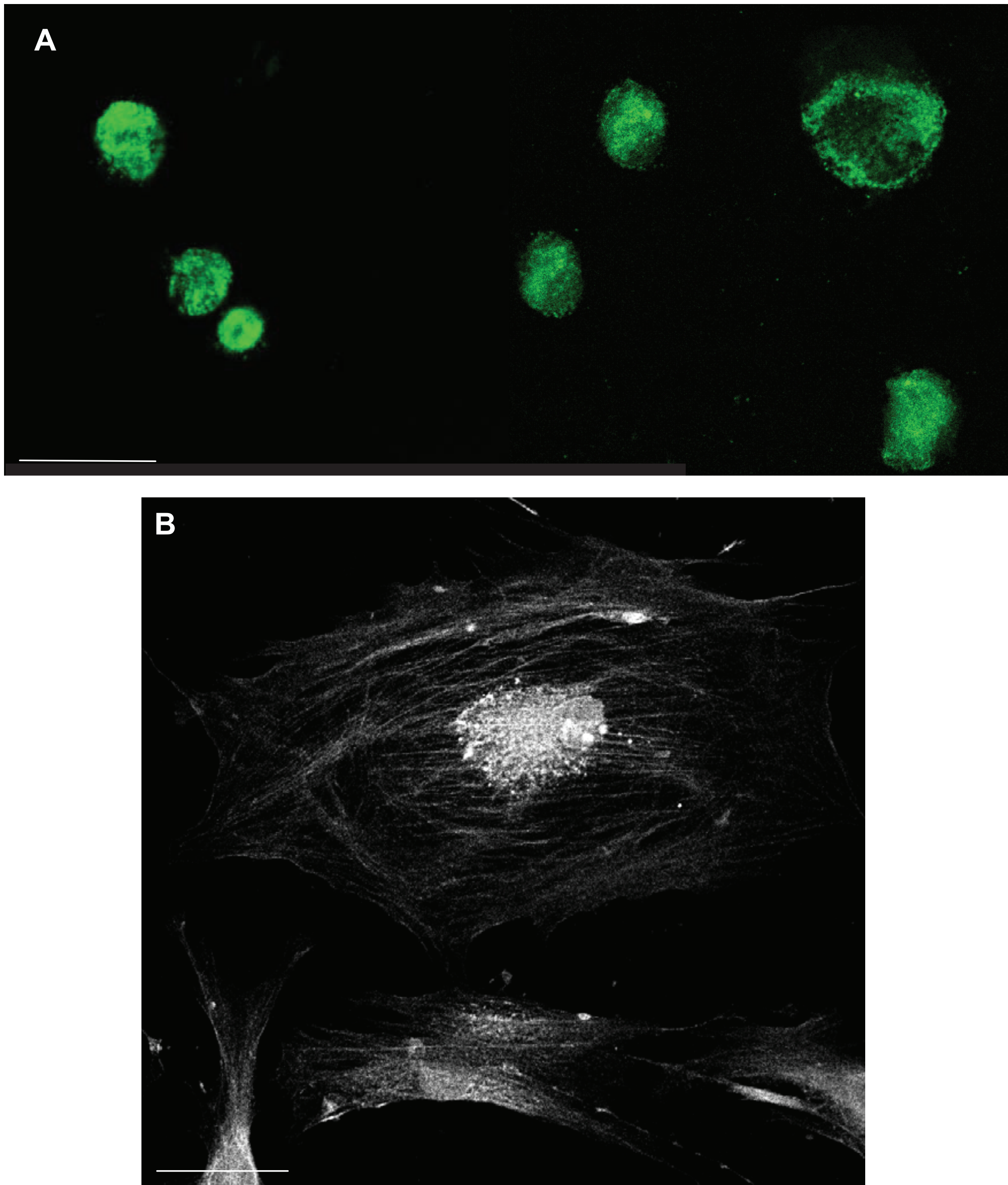


Figure 6.1. MSCs size detected by confocal microscope. (A) Size patterning of MSCs in suspension. (B) Adherent MSCs dimensions. Scale bars = 50 µm.

Our intravital microscopy study revealed the relative large size of MSCs might influence the intravascular activity in SCID mouse. After MSCs injection blood velocity significantly reduced in a cell density dependent manner until the microcirculation stopped.

In several animals the blood flow was stopped and it was not possible to perform more than one injection due to vascular occlusion and subsequent animal death. Capillaries with MSCs arrest were found. Thrombus formation was detected in arterioles and venules of the living animals due to MSCs obstructing the circulation (Figure 6.2). In some animals arterial radius (luminal dimension) was changed followed thrombosis events. Entrapments were also detectable in the lungs.

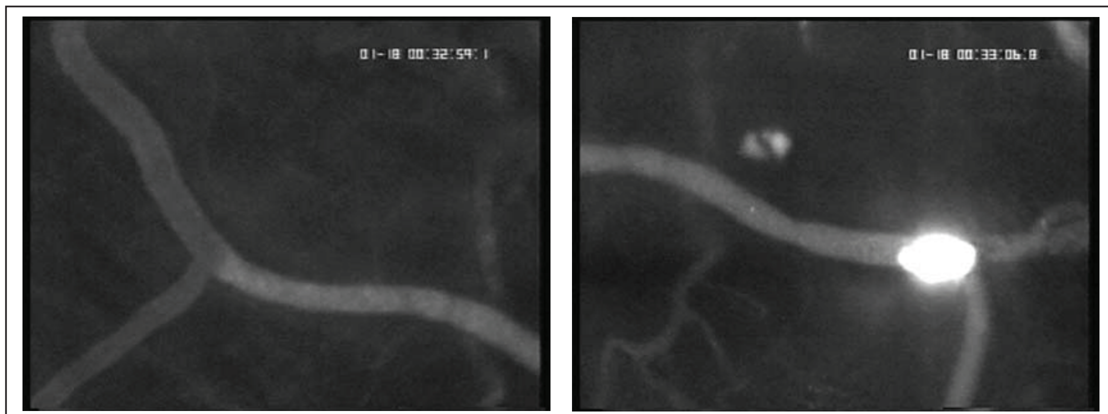


Figure 6.2. MSCs labeled with carboxy-fluorescein diacetate, succinimidyl ester (CFDA SE) impede the microcirculation. Thrombus formation in arteriole.

Our findings are consistent with the results of other groups that demonstrated systemic delivery of bone marrow derived MSCs was limited by the entrapment of cells mainly in the lungs [297] as well as liver and spleen [266]. Barbash *et al*. explained the high pulmonary entrapment of systemically infused cells due to their large size [297]. In the study of Walczak *et al*., the measured MSCs size ranged between 20-50 µm and although cells were able to bypass the endovascular barrier, entrapment in vasculature was found in 17% of the animals indicating clear risk of vascular occlusion [288]. In the study of Vulliet *et al*., 0.5 x 10^6 cells per body kg were enough to cause myocardial infarction, even in healthy vasculature [291]. Briefly, evidence suggests that the intracoronary delivery of MSCs might cause microvascular plugging and consequent no-reflow phenomena with high probability [290]. In our study, delivery of amount of injected cells was in limits in concordance with the doses used in the literature [290]

[291] [266]. To our knowledge, the present study is the first kinetic investigation of the potential adverse behavior of MSCs in the vasculature with the intravital microscopy.

The findings of reduced blood flow by angiography as well as the evidence of microvascular plugging should alert clinicians to a potential limitation of systemic or intraarterial delivery of MSCs. Following intraarterial transplantation, cells causing embolism in mouse might with a high probability lead to the same sequence of events in clinical setting. Smaller cells are able to return through muscular venules; however some of them may be entrapped in the lungs and lead to pulmonary embolism, and other undesirable consequences [attached manuscript 3].

6.2. Intravenous infusion

Intravenous infusion has been used in a number of experimental models where delivery of EPCs or MSCs has been shown to improve cardiac function after acute MI [234] [298] [299]. However, homing of cells to organs other than the heart reduces the clinical applicability of this approach [297] [300]. Indeed, it has been shown in a study of post acute MI patients that BMMN cells homing was only achieved after intracoronary stop-flow delivery (transient balloon inflations to maximize the contact time of the cells with the microcirculation of the infarct-related artery) but not after intravenous application [301].

6.3. Direct injection in the ventricular wall

Direct administration of cells into the ventricular wall might be the preferable route in case the patients present an occluded coronary artery which precludes transvascular cell delivery (patients with chronic myocardial ischemia) or when cell homing signals are not expressed at sufficient levels because of diffuse fibrosis. However, direct injection of cells into injured myocardium could create groups of isolated cells with inadequate blood supply with consequent poor cell survival [302]. The direct administration method could be suitable for the application of large cells, such as MSCs or myoblasts, which may lead to microembolization after intracoronary injection [8].

7. Safety of stem cell treatments

7.1. How to control a highly potent cell?

The novel discovery of somatic cell reprogramming into a pluripotent cell by a relatively simple genetic manipulation [179] and the growing knowledge about the closed relations which exist between stem cell and cancer [303] [304] suggest that the barriers between: a differentiated cell, a stem cell and a cancer cell might be rather easy to be overcome. In numerous studies, it has been uncovered that stem cells could give origin to cancer [132] [157] [158] [159] [167] therefore precautions to assure the safety of stem cell treatments must be taken in serious consideration. Rigorous protocols for the cell manipulation, quality controls before transplantation and long-term side effect evaluation are highly needed to ensure the security of stem cell therapy [attached manuscript 4].

7.2. Mesenchymal stem cell and cancer

As said in chapter 3, mesenchymal stem cells are self-renewing, clonal precursors of non-hematopoietic stromal tissues [102] [159] [305] [306]. Their excellent proliferation capacity makes culture expansion of MSCs an attractive strategy to generate large number of cells for autologous stem cell therapy [152] [307]. However, MSCs expansion may accumulate the deleterious mutations, resulting in spontaneous immortalization and malignant transformation. Indeed, the spontaneous transformation of MSCs after expansion culture is reported in adipose tissue-derived human MSCs [157] and bone marrow-derived mouse MSCs [158] [159] [160] [161]. The transformed MSCs are associated with phenotypic and genotypic alterations, including rapid cell proliferation and loss of contact inhibition, accumulated chromosomal instability, gradual elevation of telomerase activity and enhanced c-myc gene expression.

Further aim of the thesis was to report that rat bone marrow-derived MSCs could undergo spontaneous transformation in early passage culture. The therapeutic effects of transformed MSCs on the cardiac function were investigated in a rat left anterior descending (LAD) ligation model after intracardiac injections [attached manuscript 4].

Our study demonstrated that MSCs isolated from bone marrow of Lewis rats according to standard protocols and cultured under standard conditions may undergo

spontaneous transformation even at early passage. Inheritable changes in cells were observed, as manifested by changes in chromosomes number and alterations in metabolic features (Figure 7.1) and cell surface properties. These results were observed independently by three researchers working two years apart. Therefore, the idiosyncratic effects of a particular lot of serum in culture medium as the possible reason to induce the transformation can be excluded.

The resulting clones showed chromosomal instability by passage 3 and lost some characteristics of the phenotype of MSCs. We observed the wide variation in chromosome number, which indicate chromosome instability and may contribute to cancer initiation (Figure 7.2). Consistently, the analysis of their metabolism detected an atypical increased rate of proliferation. It is still not clear whether the increase of chromosome number occurred is due to cell fusion or chromosome replication. In fact, there are conflict evidences: the fusion of mouse bone marrow-derived cells with host cells [308] [309] [310] and the fusion of human MSCs with co-cultured epithelial cells [311] were reported; Zhou *et al.* did not detect the fusion [161]. This issue needs to be further addressed.

Zhou *et al*. [161], Tolar *et al.* [160] and Aguilar *et al.* [158] found bone marrow derived mouse MSCs showed cytogenetic aberrations after several passages in vitro culture. We also found that rat MSCs showed chromosomal instability after 3 passages. However, in our study, no sarcomas were detected 4 weeks after intravenous infusion of transformed rat MSCs into nude mice. This is different from the reports that 3 weeks after intravenous infusion of transformed mouse MSCs induced malignant sarcomas in immunodeficient mice [158] [159] [160] [161]. Recent study from Li *et al*. [312] could be one of the explanations for this discrepancy, which suggested that transformed MSCs might restore a non-malignant phenotype after fusion with host cells. It is important to further address how the cytogenetic aberrations acquired in culture correlate with the tumor initiation and progression in vivo.

We also noted several recent reports indicating that the spontaneous transformation of MSCs could occur on both bone marrow derived mouse MSCs and adipose tissue-derived human MSCs [157] [159] [161]. Hence, this phenomenon cannot be regarded as a casual phenomenon. The exploration of genetic alterations and molecular mechanisms underlying the transformation may shed a new light on regulating the process of *ex vivo* expansion of MSCs, which ensures their sustainable propagation without alterations in their genetic traits and functional degeneration.

The clinical importance of this study is related to the clinical trials that administrate human MSCs to the patients with ischemic cardiovascular diseases for regenerating cardiac functions. The present study showed that the transplantation of transformed MSCs into the infarcted hearts (Figure 7.3) revealed no cardiac function improvement as characterized by infarct size, ejection fraction, cardiac output, stroke work, stroke volume from left ventricles of the rats. Further, *ex vivo* expansion of human MSCs may induce transformation and increase the risk of cancer formation after transplantation.

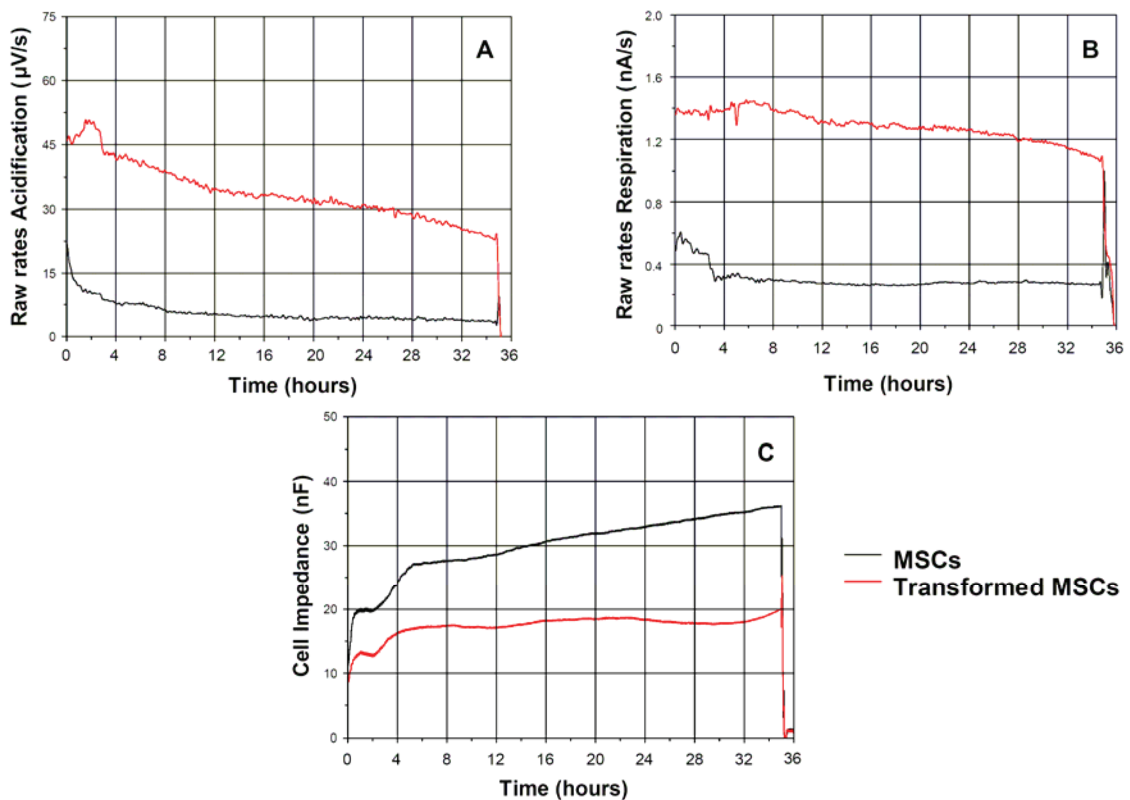


Figure 7.1. Detected signals of metabolic activities by Bionas®2500 analyzing system. (A) Acidification activity. (B) Oxygen consumption. (C) Non-standardized rates of cell impedance.

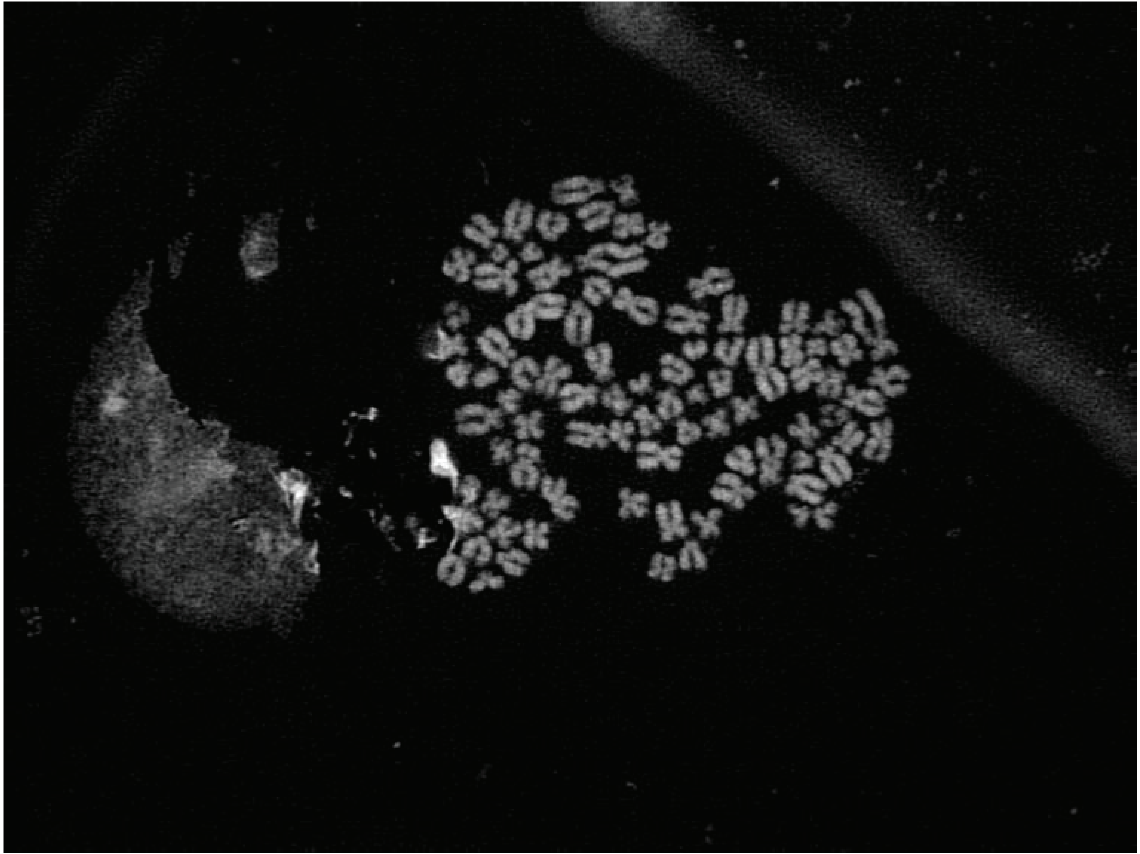


Figure 7.2. Chromosome number analysis of abnormal rat MSCs.

7.3. Mesenchymal stem cells quality control

The present study strengthens the established notion that standardized protocol for phenotypic and genotypic characterization of MSCs expanded *ex vivo* will be indispensable for the efficacy and safety considerations in clinical applications [313] [314]. Hence, systematic characterization, standardized, rigorously tested protocol and quality controls are highly valued.

In summary, we have confirmed that expansion culture of bone marrow derived rat MSCs may induce their immortalization and spontaneous transformation. The transplantation of transformed MSCs into infarcted hearts has no therapeutic effect on the cardiac functional improvement. Development of Good Manufacturing Practice (GMP) compliant culture conditions for MSCs will be of primary importance.

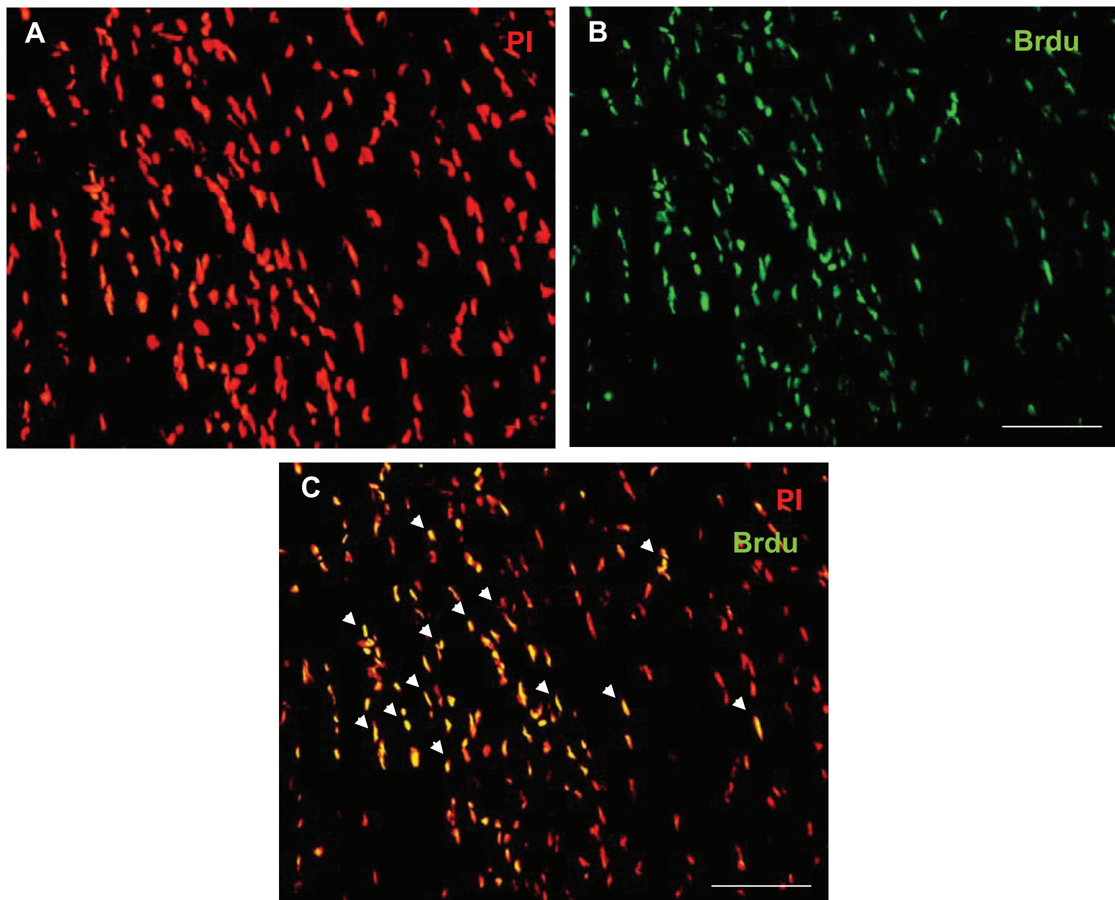


Figure 7.3. Immunohistological stain ing o f infarcted r at myocardium following MSCs injection usin g monoclonal antibodies against B rdU. (A, B) Six weeks after transplantation, sections near the infarct zone were double-stained for nuclei (PI staining) and BrdU (A: Alexa-488 labeled). (C) Merged image of double staining of sections for BrdU and nuclei. Scale bars: 200 µm.

8. Conclusion

In the present thesis, we provided novel information to the field of stem cell cardiac therapy underlining new possible application in the clinical setting. We suggested EPO application during coronary interventions or cardiac surgery might be a highly favourable treatment to promote early cardiac protection and stem cell activation. Our findings have evident translational implications for the handling of acute coronary syndrome.

In addition, we persist alerting the scientific community on the potential risks of the *ex vivo* manipulation of mesenchymal stem cell. Our experience demonstrates that MSCs therapy efficiency for myocardial infarction could be critically compromised by the appearance of cell immortalization and spontaneous transformation after culture. Thus, systematic cell characterization, standardized, rigorously tested isolation and culture protocol and quality controls are indispensable practices for stem cell transplantation approach.

Our intravital microscopy study on mesenchymal stem cell kinetics after transplantation warns clinicians about the concrete limit of systemic or intra arterial delivery of MSCs.

The ultimate translation of stem cell technologies into clinical practice will necessitate of profound understanding of the pro and contra which characterize stem cell based therapies. Most likely, the standardized clinical practice will not only be based on the knowledge of which is the best stem cell type (combined with its optimal transplantation route to treat a specific targeted disease) but also on the knowhow of the barriers that prevent cardiac regeneration.

Stem cell treatment for myocardial infarction will be probably accompanied by additional drugs (possibly derived from the uncovering of the stem cell secretion patterns or from well known medicines like EPO) to overcome hurdles caused by the hostile environment of wounded tissue (inflammation, inadequate angiogenesis and fibrosis).

Until now, clinical trials have been focused on the use of cell types which are easy to be isolated (e.g. bone marrow mononuclear cells and endothelial progenitors) but perhaps these cell types do not represent the best population committed to cardiac regeneration. Additional understanding of cardiomyocyte development and turnover (in

natural conditions and after injury) will be a necessary step forward for the establishment of stem cell based therapies.

Finally, an accurate evaluation of the safety of stem cell based treatments with the development of standardized, well characterized cell type isolation and manipulation without adverse tumorigenesis will be essential.

9. References

[1] Tamburino C, Angiolillo DJ, Capranzano P, Di Salvo M, Ussia G, La Manna A, *et al.* Long-term clinical outcomes after drug-eluting stent implantation in unprotected left main coronary artery disease. Catheter Cardiovasc Interv 2009;73:291-298.

[2] Veselka J, Cerna D, Zimolova P, Martinkovicova L, Fiedler J, Hajek P, *et al.* Feasibility, safety, and early outcomes of direct carotid artery stent implantation with use of the FilterWire EZtrade mark Embolic Protection System. Catheter Cardiovasc Interv 2008.

[3] Mylotte D, Byrne RA, Iijima R, Kastrati A. Multivessel percutaneous coronary intervention: a review of the literature and fallacies in its interpretation. Minerva Cardioangiol 2008;56:493-510.

[4] Stamm C, Westphal B, Kleine HD, Petzsch M, Kittner C, Klinge H, *et al.* Autologous bone-marrow stem-cell transplantation for myocardial regeneration. Lancet 2003;361:45-46.

[5] Kaminski A, Steinhoff G. Current status of intramyocardial bone marrow stem cell transplantation. Semin Thorac Cardiovasc Surg 2008;20:119-125.

[6] Murasawa S, Asahara T. Cardiogenic potential of endothelial progenitor cells. Ther Adv Cardiovasc Dis 2008;2:341-348.

[7] Breitbach M, Bostani T, Roell W, Xia Y, Dewald O, Nygren JM, *et al.* Potential risks of bone marrow cell transplantation into infarcted hearts. Blood 2007;110:1362-1369.

[8] Wollert KC, Drexler H. Clinical applications of stem cells for the heart. Circ Res 2005;96:151-163.

[9] Dimmeler S, Zeiher AM, Schneider MD. Unchain my heart: the scientific foundations of cardiac repair. J Clin Invest 2005;115:572-583.

[10] Maekawa T, Okada A, Kawanami C, Matsushima Y, Asahara M, Idei Y, *et al.* [A case of nutcracker esophagus associated with ischemic heart disease]. Nippon Shokakibyo Gakkai Zasshi 1996;93:347-350.

[11] Hori M, Nishida K. Toll-like receptor signaling: defensive or offensive for the heart? Circ Res 2008;102:137-139.

[12] Yndestad A, Damas JK, Oie E, Ueland T, Gullestad L, Aukrust P. Systemic inflammation in heart failure--the whys and wherefores. Heart Fail Rev 2006;11:83-92.

[13] Wenger NK, Helmy T, Khan BV, Patel AD. Evidence-based management of acute myocardial infarction in the elderly--current perspectives. MedGenMed 2005;7:73.

[14] Braunwald E. Control of myocardial oxygen consumption: physiologic and clinical considerations. Am J Cardiol 1971;27:416-432.

[15] Ferrans VJ, Hibbs RG, Cipriano PR, Buja LM. Histochemical and electron microscopic studies of norepinephrine-induced myocardial necrosis in rats. Recent Adv Stud Cardiac Struct Metab 1972;1:495-525.

[16] Reimer KA, Lowe JE, Rasmussen MM, Jennings RB. The wavefront phenomenon of ischemic cell death. 1. Myocardial infarct size vs duration of coronary occlusion in dogs. Circulation 1977;56:786-794.

[17] Alpert JS, Thygesen K, Antman E, Bassand JP. Myocardial infarction redefined--a consensus document of The Joint European Society of Cardiology/American

College of Cardiology Committee for the redefinition of myocardial infarction. J Am Coll Cardiol 2000;36:959-969.
[18] Hirayama A, Adachi T, Asada S, Mishima M, Nanto S, Kusuoka H, *et al.* Late reperfusion for acute myocardial infarction limits the dilatation of left ventricle without the reduction of infarct size. Circulation 1993;88:2565-2574.
[19] Kapadia S, Lee J, Torre-Amione G, Birdsall HH, Ma TS, Mann DL. Tumor necrosis factor-alpha gene and protein expression in adult feline myocardium after endotoxin administration. J Clin Invest 1995;96:1042-1052.
[20] Kapadia SR, Oral H, Lee J, Nakano M, Taffet GE, Mann DL. Hemodynamic regulation of tumor necrosis factor-alpha gene and protein expression in adult feline myocardium. Circ Res 1997;81:187-195.
[21] Mann DL. Stress-activated cytokines and the heart: from adaptation to maladaptation. Annu Rev Physiol 2003;65:81-101.
[22] Nian M, Lee P, Khaper N, Liu P. Inflammatory cytokines and postmyocardial infarction remodeling. Circ Res 2004;94:1543-1553.
[23] Ono K, Matsumori A, Shioi T, Furukawa Y, Sasayama S. Cytokine gene expression after myocardial infarction in rat hearts: possible implication in left ventricular remodeling. Circulation 1998;98:149-156.
[24] Deten A, Volz HC, Briest W, Zimmer HG. Cardiac cytokine expression is upregulated in the acute phase after myocardial infarction. Experimental studies in rats. Cardiovasc Res 2002;55:329-340.
[25] Irwin MW, Mak S, Mann DL, Qu R, Penninger JM, Yan A, *et al.* Tissue expression and immunolocalization of tumor necrosis factor-alpha in postinfarction dysfunctional myocardium. Circulation 1999;99:1492-1498.
[26] Beg AA, Baltimore D. An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. Science 1996;274:782-784.
[27] Murakami M, Hibi M, Nakagawa N, Nakagawa T, Yasukawa K, Yamanishi K, *et al.* IL-6-induced homodimerization of gp130 and associated activation of a tyrosine kinase. Science 1993;260:1808-1810.
[28] Hirota H, Yoshida K, Kishimoto T, Taga T. Continuous activation of gp130, a signal-transducing receptor component for interleukin 6-related cytokines, causes myocardial hypertrophy in mice. Proc Natl Acad Sci U S A 1995;92:4862-4866.
[29] Shiomi T, Tsutsui H, Hayashidani S, Suematsu N, Ikeuchi M, Wen J, *et al.* Pioglitazone, a peroxisome proliferator-activated receptor-gamma agonist, attenuates left ventricular remodeling and failure after experimental myocardial infarction. Circulation 2002;106:3126-3132.
[30] Yue Tl TL, Chen J, Bao W, Narayanan PK, Bril A, Jiang W, *et al.* In vivo myocardial protection from ischemia/reperfusion injury by the peroxisome proliferator-activated receptor-gamma agonist rosiglitazone. Circulation 2001;104:2588-2594.
[31] Yao YJ, Geng DF, Wang JF, Yang MH, Zhang YL, Nie RQ, *et al.* [PPAR gamma agonist rosiglitazone alleviates hypoxia/reoxygenation-induced oxidative stress and apoptosis in rat cardiac myocytes.]. Nan Fang Yi Ke Da Xue Xue Bao 2009;29:689-693.
[32] Liu HR, Tao L, Gao E, Qu Y, Lau WB, Lopez BL, *et al.* Rosiglitazone inhibits hypercholesterolaemia-induced myeloperoxidase upregulation--a novel mechanism for the cardioprotective effects of PPAR agonists. Cardiovasc Res 2009;81:344-352.

[33] Meldrum DR, Dinarello CA, Cleveland JC, Jr., Cain BS, Shames BD, Meng X, *et al.* Hydrogen peroxide induces tumor necrosis factor alpha- mediated cardiac injury by a P38 mitogen-activated protein kinase-dependent mechanism. Surgery 1998;124:291-296; discussion 297.

[34] Nakamura K, Fushimi K, Kouchi H, Mihara K, Miyazaki M, Ohe T, *et al.* Inhibitory effects of antioxidants on neonatal rat cardiac myocyte hypertrophy induced by tumor necrosis factor-alpha and angiotensin II. Circulation 1998;98:794-799.

[35] Nakao N, Kurokawa T, Nonami T, Tumurkhuu G, Koide N, Yokochi T. Hydrogen peroxide induces the production of tumor necrosis factor-alpha in RAW 264.7 macrophage cells via activation of p38 and stress- activated protein kinase. Innate Immun 2008;14:190-196.

[36] Tang D, Shi Y, Kang R, Li T, Xiao W, Wang H, *et al.* Hydrogen peroxide stimulates macrophages and monocytes to actively release HMGB1. J Leukoc Biol 2007;81:741-747.

[37] van Zoelen MA, Yang H, Florquin S, Meijers JC, Akira S, Arnold B, *et al.* Role of toll-like receptors 2 and 4, and the receptor for advanced glycation end products in high-mobility group box 1-induced inflammation in vivo. Shock 2009;31:280-284.

[38] Chao W. Toll-like receptor signaling: a critical modulator of cell survival and ischemic injury in the heart. Am J Physiol Heart Circ Physiol 2009;296:H1-12.

[39] Yan SF, Ramasamy R, Schmidt AM. The receptor for advanced glycation endproducts (RAGE) and cardiovascular disease. Expert Rev Mol Med 2009;11:e9.

[40] Ojaniemi M, Glumoff V, Harju K, Liljeroos M, Vuori K, Hallman M. Phosphatidylinositol 3-kinase is involved in Toll-like receptor 4-mediated cytokine expression in mouse macrophages. Eur J Immunol 2003;33:597-605.

[41] Kawai T, Akira S. TLR signaling. Semin Immunol 2007;19:24-32.

[42] Frantz S, Ertl G, Bauersachs J. Mechanisms of disease: Toll-like receptors in cardiovascular disease. Nat Clin Pract Cardiovasc Med 2007;4:444-454.

[43] Bae YS, Lee JH, Choi SH, Kim S, Almazan F, Witztum JL, *et al.* Macrophages generate reactive oxygen species in response to minimally oxidized low-density lipoprotein: toll-like receptor 4- and spleen tyrosine kinase-dependent activation of NADPH oxidase 2. Circ Res 2009;104:210-218, 221p following 218.

[44] Yan SF, D'Agati V, Schmidt AM, Ramasamy R. Receptor for Advanced Glycation Endproducts (RAGE): a formidable force in the pathogenesis of the cardiovascular complications of diabetes & aging. Curr Mol Med 2007;7:699-710.

[45] Kumar AG, Ballantyne CM, Michael LH, Kukielka GL, Youker KA, Lindsey ML, *et al.* Induction of monocyte chemoattractant protein-1 in the small veins of the ischemic and reperfused canine myocardium. Circulation 1997;95:693-700.

[46] Kakio T, Matsumori A, Ono K, Ito H, Matsushima K, Sasayama S. Roles and relationship of macrophages and monocyte chemotactic and activating factor/monocyte chemoattractant protein-1 in the ischemic and reperfused rat heart. Lab Invest 2000;80:1127-1136.

[47] Ebnet K, Vestweber D. Molecular mechanisms that control leukocyte extravasation: the selectins and the chemokines. Histochem Cell Biol 1999;112:1-23.

[48] Frangogiannis NG, Perrard JL, Mendoza LH, Burns AR, Lindsey ML, Ballantyne CM, *et al.* Stem cell factor induction is associated with mast cell

accumulation after canine myocardial ischemia and reperfusion. Circulation 1998;98:687-698.

[49] Garcia-Dorado D, Theroux P, Duran JM, Solares J, Alonso J, Sanz E, *et al.* Selective inhibition of the contractile apparatus. A new approach to modification of infarct size, infarct composition, and infarct geometry during coronary artery occlusion and reperfusion. Circulation 1992;85:1160-1174.

[50] Ruiz-Meana M, Garcia-Dorado D. Translational cardiovascular medicine (II). Pathophysiology of ischemia-reperfusion injury: new therapeutic options for acute myocardial infarction. Rev Esp Cardiol 2009;62:199-209.

[51] Hearse DJ, Humphrey SM, Chain EB. Abrupt reoxygenation of the anoxic potassium-arrested perfused rat heart: a study of myocardial enzyme release. J Mol Cell Cardiol 1973;5:395-407.

[52] Vander Heide RS, Angelo JP, Altschuld RA, Ganote CE. Energy dependence of contraction band formation in perfused hearts and isolated adult myocytes. Am J Pathol 1986;125:55-68.

[53] Piper HM, Garcia-Dorado D, Ovize M. A fresh look at reperfusion injury. Cardiovasc Res 1998;38:291-300.

[54] Barrabes JA, Garcia-Dorado D, Ruiz-Meana M, Piper HM, Solares J, Gonzalez MA, *et al.* Myocardial segment shrinkage during coronary reperfusion in situ. Relation to hypercontracture and myocardial necrosis. Pflugers Arch 1996;431:519-526.

[55] Siegmund B, Zude R, Piper HM. Recovery of anoxic-reoxygenated cardiomyocytes from severe Ca2+ overload. Am J Physiol 1992;263:H1262-1269.

[56] Inserte J, Garcia-Dorado D, Ruiz-Meana M, Padilla F, Barrabes JA, Pina P, *et al.* Effect of inhibition of Na(+)/Ca(2+) exchanger at the time of myocardial reperfusion on hypercontracture and cell death. Cardiovasc Res 2002;55:739-748.

[57] Garcia-Dorado D, Theroux P, Munoz R, Alonso J, Elizaga J, Fernandez-Aviles F, *et al.* Favorable effects of hyperosmotic reperfusion on myocardial edema and infarct size. Am J Physiol 1992;262:H17-22.

[58] Inserte J, Garcia-Dorado D, Hernando V, Soler-Soler J. Calpain-mediated impairment of Na+/K+-ATPase activity during early reperfusion contributes to cell death after myocardial ischemia. Circ Res 2005;97:465-473.

[59] Inserte J, Garcia-Dorado D, Hernando V, Barba I, Soler-Soler J. Ischemic preconditioning prevents calpain-mediated impairment of Na+/K+-ATPase activity during early reperfusion. Cardiovasc Res 2006;70:364-373.

[60] Inserte J, Garcia-Dorado D, Ruiz-Meana M, Agullo L, Pina P, Soler-Soler J. Ischemic preconditioning attenuates calpain-mediated degradation of structural proteins through a protein kinase A-dependent mechanism. Cardiovasc Res 2004;64:105-114.

[61] Barrabes JA, Garcia-Dorado D, Mirabet M, Inserte J, Agullo L, Soriano B, *et al.* Antagonism of selectin function attenuates microvascular platelet deposition and platelet-mediated myocardial injury after transient ischemia. J Am Coll Cardiol 2005;45:293-299.

[62] Mirabet M, Garcia-Dorado D, Ruiz-Meana M, Barrabes JA, Soler-Soler J. Thrombin increases cardiomyocyte acute cell death after ischemia and reperfusion. J Mol Cell Cardiol 2005;39:277-283.

[63] Barrabes JA, Mirabet M, Agullo L, Figueras J, Pizcueta P, Garcia-Dorado D. Platelet deposition in remote cardiac regions after coronary occlusion. Eur J Clin Invest 2007;37:939-946.

[64] Morgan EN, Boyle EM, Jr., Yun W, Griscavage-Ennis JM, Farr AL, Canty TG, Jr., *et al.* An essential role for NF-kappaB in the cardioadaptive response to ischemia. Ann Thorac Surg 1999;68:377-382.

[65] Chandrasekar B, Freeman GL. Induction of nuclear factor kappaB and activation protein 1 in postischemic myocardium. FEBS Lett 1997;401:30-34.

[66] Morishita R, Sugimoto T, Aoki M, Kida I, Tomita N, Moriguchi A*, et al.* In vivo transfection of cis element "decoy" against nuclear factor-kappaB binding site prevents myocardial infarction. Nat Med 1997;3:894-899.

[67] Frangogiannis NG. Targeting the inflammatory response in healing myocardial infarcts. Curr Med Chem 2006;13:1877-1893.

[68] Opie LH, Commerford PJ, Gersh BJ, Pfeffer MA. Controversies in ventricular remodelling. Lancet 2006;367:356-367.

[69] White HD, Norris RM, Brown MA, Brandt PW, Whitlock RM, Wild CJ. Left ventricular end-systolic volume as the major determinant of survival after recovery from myocardial infarction. Circulation 1987;76:44-51.

[70] St John Sutton M, Pfeffer MA, Plappert T, Rouleau JL, Moye LA, Dagenais GR*, et al.* Quantitative two-dimensional echocardiographic measurements are major predictors of adverse cardiovascular events after acute myocardial infarction. The protective effects of captopril. Circulation 1994;89:68-75.

[71] McKay RG, Pfeffer MA, Pasternak RC, Markis JE, Come PC, Nakao S*, et al.* Left ventricular remodeling after myocardial infarction: a corollary to infarct expansion. Circulation 1986;74:693-702.

[72] Ertl G, Frantz S. Healing after myocardial infarction. Cardiovasc Res 2005;66:22-32.

[73] Frantz S, Bauersachs J, Ertl G. Post-infarct remodelling: contribution of wound healing and inflammation. Cardiovasc Res 2009;81:474-481.

[74] Palojoki E, Saraste A, Eriksson A, Pulkki K, Kallajoki M, Voipio-Pulkki LM*, et al.* Cardiomyocyte apoptosis and ventricular remodeling after myocardial infarction in rats. Am J Physiol Heart Circ Physiol 2001;280:H2726-2731.

[75] Krown KA, Page MT, Nguyen C, Zechner D, Gutierrez V, Comstock KL*, et al.* Tumor necrosis factor alpha-induced apoptosis in cardiac myocytes. Involvement of the sphingolipid signaling cascade in cardiac cell death. J Clin Invest 1996;98:2854-2865.

[76] Yokoyama T, Nakano M, Bednarczyk JL, McIntyre BW, Entman M, Mann DL. Tumor necrosis factor-alpha provokes a hypertrophic growth response in adult cardiac myocytes. Circulation 1997;95:1247-1252.

[77] Kubota T, McTiernan CF, Frye CS, Slawson SE, Lemster BH, Koretsky AP*, et al.* Dilated cardiomyopathy in transgenic mice with cardiac-specific overexpression of tumor necrosis factor-alpha. Circ Res 1997;81:627-635.

[78] Frangogiannis NG, Smith CW, Entman ML. The inflammatory response in myocardial infarction. Cardiovasc Res 2002;53:31-47.

[79] Hirano T. Interleukin 6 and its receptor: ten years later. Int Rev Immunol 1998;16:249-284.

[80] Gallucci RM, Simeonova PP, Matheson JM, Kommineni C, Guriel JL, Sugawara T*, et al.* Impaired cutaneous wound healing in interleukin-6-deficient and immunosuppressed mice. FASEB J 2000;14:2525-2531.

[81] Yokoyama T, Vaca L, Rossen RD, Durante W, Hazarika P, Mann DL. Cellular basis for the negative inotropic effects of tumor necrosis factor-alpha in the adult mammalian heart. J Clin Invest 1993;92:2303-2312.
[82] Goldhaber JI. Free radicals enhance Na+/Ca2+ exchange in ventricular myocytes. Am J Physiol 1996;271:H823-833.
[83] Ferdinandy P, Danial H, Ambrus I, Rothery RA, Schulz R. Peroxynitrite is a major contributor to cytokine-induced myocardial contractile failure. Circ Res 2000;87:241-247.
[84] Suematsu N, Tsutsui H, Wen J, Kang D, Ikeuchi M, Ide T, *et al.* Oxidative stress mediates tumor necrosis factor-alpha-induced mitochondrial DNA damage and dysfunction in cardiac myocytes. Circulation 2003;107:1418-1423.
[85] Sia YT, Parker TG, Liu P, Tsoporis JN, Adam A, Rouleau JL. Improved post-myocardial infarction survival with probucol in rats: effects on left ventricular function, morphology, cardiac oxidative stress and cytokine expression. J Am Coll Cardiol 2002;39:148-156.
[86] Khaper N, Singal PK. Modulation of oxidative stress by a selective inhibition of angiotensin II type 1 receptors in MI rats. J Am Coll Cardiol 2001;37:1461-1466.
[87] Sun Y, Zhang J, Lu L, Chen SS, Quinn MT, Weber KT. Aldosterone-induced inflammation in the rat heart: role of oxidative stress. Am J Pathol 2002;161:1773-1781.
[88] Deten A, Holzl A, Leicht M, Barth W, Zimmer HG. Changes in extracellular matrix and in transforming growth factor beta isoforms after coronary artery ligation in rats. J Mol Cell Cardiol 2001;33:1191-1207.
[89] Anversa P, Nadal-Ginard B. Myocyte renewal and ventricular remodelling. Nature 2002;415:240-243.
[90] Sun M, Opavsky MA, Stewart DJ, Rabinovitch M, Dawood F, Wen WH, *et al.* Temporal response and localization of integrins beta1 and beta3 in the heart after myocardial infarction: regulation by cytokines. Circulation 2003;107:1046-1052.
[91] Bryant D, Becker L, Richardson J, Shelton J, Franco F, Peshock R, *et al.* Cardiac failure in transgenic mice with myocardial expression of tumor necrosis factor-alpha. Circulation 1998;97:1375-1381.
[92] Sivasubramanian N, Coker ML, Kurrelmeyer KM, MacLellan WR, DeMayo FJ, Spinale FG, *et al.* Left ventricular remodeling in transgenic mice with cardiac restricted overexpression of tumor necrosis factor. Circulation 2001;104:826-831.
[93] Rosenkranz S. TGF-beta1 and angiotensin networking in cardiac remodeling. Cardiovasc Res 2004;63:423-432.
[94] Creemers EE, Cleutjens JP, Smits JF, Daemen MJ. Matrix metalloproteinase inhibition after myocardial infarction: a new approach to prevent heart failure? Circ Res 2001;89:201-210.
[95] Siwik DA, Chang DL, Colucci WS. Interleukin-1beta and tumor necrosis factor-alpha decrease collagen synthesis and increase matrix metalloproteinase activity in cardiac fibroblasts in vitro. Circ Res 2000;86:1259-1265.
[96] Spinale FG, Coker ML, Heung LJ, Bond BR, Gunasinghe HR, Etoh T, *et al.* A matrix metalloproteinase induction/activation system exists in the human left ventricular myocardium and is upregulated in heart failure. Circulation 2000;102:1944-1949.

[97] Peng J, Gurantz D, Tran V, Cowling RT, Greenberg BH. Tumor necrosis factor-alpha-induced AT1 receptor upregulation enhances angiotensin II-mediated cardiac fibroblast responses that favor fibrosis. Circ Res 2002;91:1119-1126.
[98] Trueblood NA, Xie Z, Communal C, Sam F, Ngoy S, Liaw L, *et al.* Exaggerated left ventricular dilation and reduced collagen deposition after myocardial infarction in mice lacking osteopontin. Circ Res 2001;88:1080-1087.
[99] Xie Z, Singh M, Siwik DA, Joyner WL, Singh K. Osteopontin inhibits interleukin-1beta-stimulated increases in matrix metalloproteinase activity in adult rat cardiac fibroblasts: role of protein kinase C-zeta. J Biol Chem 2003;278:48546-48552.
[100] Takahashi K, Fukushima S, Yamahara K, Yashiro K, Shintani Y, Coppen SR, *et al.* Modulated inflammation by injection of high-mobility group box 1 recovers post-infarction chronically failing heart. Circulation 2008;118:S106-114.
[101] Li TS, Hayashi M, Ito H, Furutani A, Murata T, Matsuzaki M, *et al.* Regeneration of infarcted myocardium by intramyocardial implantation of ex vivo transforming growth factor-beta-preprogrammed bone marrow stem cells. Circulation 2005;111:2438-2445.
[102] Li W, Ma N, Ong LL, Nesselmann C, Klopsch C, Ladilov Y, *et al.* Bcl-2 engineered MSCs inhibited apoptosis and improved heart function. Stem Cells 2007;25:2118-2127.
[103] Heba G, Krzeminski T, Porc M, Grzyb J, Dembinska-Kiec A. Relation between expression of TNF alpha, iNOS, VEGF mRNA and development of heart failure after experimental myocardial infarction in rats. J Physiol Pharmacol 2001;52:39-52.
[104] Tamura K, Nakajima H, Rakue H, Sasame A, Naito Y, Nagai Y, *et al.* Elevated circulating levels of basic fibroblast growth factor and vascular endothelial growth factor in patients with acute myocardial infarction. Jpn Circ J 1999;63:357-361.
[105] Pannitteri G, Petrucci E, Testa U. Coordinate release of angiogenic growth factors after acute myocardial infarction: evidence of a two-wave production. J Cardiovasc Med (Hagerstown) 2006;7:872-879.
[106] Indications for fibrinolytic therapy in suspected acute myocardial infarction: collaborative overview of early mortality and major morbidity results from all randomised trials of more than 1000 patients. Fibrinolytic Therapy Trialists' (FTT) Collaborative Group. Lancet 1994;343:311-322.
[107] Boersma E, Maas AC, Deckers JW, Simoons ML. Early thrombolytic treatment in acute myocardial infarction: reappraisal of the golden hour. Lancet 1996;348:771-775.
[108] White HD, Van de Werf FJ. Thrombolysis for acute myocardial infarction. Circulation 1998;97:1632-1646.
[109] Chesebro JH, Knatterud G, Roberts R, Borer J, Cohen LS, Dalen J, *et al.* Thrombolysis in Myocardial Infarction (TIMI) Trial, Phase I: A comparison between intravenous tissue plasminogen activator and intravenous streptokinase. Clinical findings through hospital discharge. Circulation 1987;76:142-154.
[110] Pinar E, Albarran A, Baz JA, Mauri J. [Update on interventional cardiology]. Rev Esp Cardiol 2009;62 Suppl 1:101-116.
[111] Keeley EC, Boura JA, Grines CL. Primary angioplasty versus intravenous thrombolytic therapy for acute myocardial infarction: a quantitative review of 23 randomised trials. Lancet 2003;361:13-20.

[112] Grines CL, Browne KF, Marco J, Rothbaum D, Stone GW, O'Keefe J, *et al.* A comparison of immediate angioplasty with thrombolytic therapy for acute myocardial infarction. The Primary Angioplasty in Myocardial Infarction Study Group. N Engl J Med 1993;328:673-679.
[113] A clinical trial comparing primary coronary angioplasty with tissue plasminogen activator for acute myocardial infarction. The Global Use of Strategies to Open Occluded Coronary Arteries in Acute Coronary Syndromes (GUSTO IIb) Angioplasty Substudy Investigators. N Engl J Med 1997;336:1621-1628.
[114] Grines CL, Cox DA, Stone GW, Garcia E, Mattos LA, Giambartolomei A, *et al.* Coronary angioplasty with or without stent implantation for acute myocardial infarction. Stent Primary Angioplasty in Myocardial Infarction Study Group. N Engl J Med 1999;341:1949-1956.
[115] Babaev A, Frederick PD, Pasta DJ, Every N, Sichrovsky T, Hochman JS. Trends in management and outcomes of patients with acute myocardial infarction complicated by cardiogenic shock. JAMA 2005;294:448-454.
[116] Raja SG, Haider Z, Ahmad M, Zaman H. Saphenous vein grafts: to use or not to use? Heart Lung Circ 2004;13:403-409.
[117] Hannan EL, Racz MJ, Walford G, Jones RH, Ryan TJ, Bennett E, *et al.* Long-term outcomes of coronary-artery bypass grafting versus stent implantation. N Engl J Med 2005;352:2174-2183.
[118] Bourassa MG. Clinical trials of coronary revascularization: coronary angioplasty vs. coronary bypass grafting. Curr Opin Cardiol 2000;15:281-286.
[119] Schachinger V, Erbs S, Elsasser A, Haberbosch W, Hambrecht R, Holschermann H, *et al.* Intracoronary bone marrow-derived progenitor cells in acute myocardial infarction. N Engl J Med 2006;355:1210-1221.
[120] Segers VF, Lee RT. Stem-cell therapy for cardiac disease. Nature 2008;451:937-942.
[121] Christman KL, Lee RJ. Biomaterials for the treatment of myocardial infarction. J Am Coll Cardiol 2006;48:907-913.
[122] Beltrami AP, Urbanek K, Kajstura J, Yan SM, Finato N, Bussani R, *et al.* Evidence that human cardiac myocytes divide after myocardial infarction. N Engl J Med 2001;344:1750-1757.
[123] Wagers AJ, Conboy IM. Cellular and molecular signatures of muscle regeneration: current concepts and controversies in adult myogenesis. Cell 2005;122:659-667.
[124] Shi X, Garry DJ. Muscle stem cells in development, regeneration, and disease. Genes Dev 2006;20:1692-1708.
[125] Borchardt T, Braun T. Cardiovascular regeneration in non-mammalian model systems: what are the differences between newts and man? Thromb Haemost 2007;98:311-318.
[126] Poss KD. Getting to the heart of regeneration in zebrafish. Semin Cell Dev Biol 2007;18:36-45.
[127] Lepilina A, Coon AN, Kikuchi K, Holdway JE, Roberts RW, Burns CG, *et al.* A dynamic epicardial injury response supports progenitor cell activity during zebrafish heart regeneration. Cell 2006;127:607-619.
[128] van Amerongen MJ, Engel FB. Features of cardiomyocyte proliferation and its potential for cardiac regeneration. J Cell Mol Med 2008;12:2233-2244.
[129] Ahuja P, Sdek P, MacLellan WR. Cardiac myocyte cell cycle control in development, disease, and regeneration. Physiol Rev 2007;87:521-544.

[130] Rubart M, Field LJ. Cardiac regeneration: repopulating the heart. Annu Rev Physiol 2006;68:29-49.

[131] Engel FB, Schebesta M, Duong MT, Lu G, Ren S, Madwed JB, *et al.* p38 MAP kinase inhibition enables proliferation of adult mammalian cardiomyocytes. Genes Dev 2005;19:1175-1187.

[132] Laflamme MA, Murry CE. Regenerating the heart. Nat Biotechnol 2005;23:845-856.

[133] Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, *et al.* Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 2003;114:763-776.

[134] Laugwitz KL, Moretti A, Lam J, Gruber P, Chen Y, Woodard S, *et al.* Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. Nature 2005;433:647-653.

[135] Oh H, Bradfute SB, Gallardo TD, Nakamura T, Gaussin V, Mishina Y, *et al.* Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. Proc Natl Acad Sci U S A 2003;100:12313-12318.

[136] Hsieh PC, Segers VF, Davis ME, MacGillivray C, Gannon J, Molkentin JD, *et al.* Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury. Nat Med 2007;13:970-974.

[137] Poss KD, Wilson LG, Keating MT. Heart regeneration in zebrafish. Science 2002;298:2188-2190.

[138] Menasche P. Skeletal myoblasts as a therapeutic agent. Prog Cardiovasc Dis 2007;50:7-17.

[139] Cleland JG, Coletta AP, Abdellah AT, Nasir M, Hobson N, Freemantle N, *et al.* Clinical trials update from the American Heart Association 2006: OAT, SALT 1 and 2, MAGIC, ABCD, PABA-CHF, IMPROVE-CHF, and percutaneous mitral annuloplasty. Eur J Heart Fail 2007;9:92-97.

[140] Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, *et al.* Bone marrow cells regenerate infarcted myocardium. Nature 2001;410:701-705.

[141] Leri A, Kajstura J, Anversa P. Cardiac stem cells and mechanisms of myocardial regeneration. Physiol Rev 2005;85:1373-1416.

[142] Jackson KA, Majka SM, Wang H, Pocius J, Hartley CJ, Majesky MW, *et al.* Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J Clin Invest 2001;107:1395-1402.

[143] Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, *et al.* Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature 2004;428:664-668.

[144] Balsam LB, Wagers AJ, Christensen JL, Kofidis T, Weissman IL, Robbins RC. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature 2004;428:668-673.

[145] Stuckey DJ, Carr CA, Martin-Rendon E, Tyler DJ, Willmott C, Cassidy PJ, *et al.* Iron particles for noninvasive monitoring of bone marrow stromal cell engraftment into, and isolation of viable engrafted donor cells from, the heart. Stem Cells 2006;24:1968-1975.

[146] Young PP, Vaughan DE, Hatzopoulos AK. Biologic properties of endothelial progenitor cells and their potential for cell therapy. Prog Cardiovasc Dis 2007;49:421-429.

[147] Smith RR, Barile L, Messina E, Marban E. Stem cells in the heart: what's the buzz all about? Part 2: Arrhythmic risks and clinical studies. Heart Rhythm 2008;5:880-887.

[148] Narmoneva DA, Vukmirovic R, Davis ME, Kamm RD, Lee RT. Endothelial cells promote cardiac myocyte survival and spatial reorganization: implications for cardiac regeneration. Circulation 2004;110:962-968.
[149] Abdel-Latif A, Bolli R, Tleyjeh IM, Montori VM, Perin EC, Hornung CA*, et al.* Adult bone marrow-derived cells for cardiac repair: a systematic review and meta-analysis. Arch Intern Med 2007;167:989-997.
[150] Stamm C, Liebold A, Steinhoff G, Strunk D. Stem cell therapy for ischemic heart disease: beginning or end of the road? Cell Transplant 2006;15 Suppl 1:S47-56.
[151] Timmermans F, Plum J, Yoder MC, Ingram DA, Vandekerckhove B, Case J. Endothelial progenitor cells: identity defined? J Cell Mol Med 2009;13:87-102.
[152] Bianco P, Riminucci M, Gronthos S, Robey PG. Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells 2001;19:180-192.
[153] Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. J Cell Biochem 2006;98:1076-1084.
[154] Miyahara Y, Nagaya N, Kataoka M, Yanagawa B, Tanaka K, Hao H*, et al.* Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. Nat Med 2006;12:459-465.
[155] Amado LC, Saliaris AP, Schuleri KH, St John M, Xie JS, Cattaneo S*, et al.* Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells after myocardial infarction. Proc Natl Acad Sci U S A 2005;102:11474-11479.
[156] Gnecchi M, He H, Noiseux N, Liang OD, Zhang L, Morello F*, et al.* Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. FASEB J 2006;20:661-669.
[157] Rubio D, Garcia-Castro J, Martin MC, de la Fuente R, Cigudosa JC, Lloyd AC*, et al.* Spontaneous human adult stem cell transformation. Cancer Res 2005;65:3035-3039.
[158] Aguilar S, Nye E, Chan J, Loebinger M, Spencer-Dene B, Fisk N*, et al.* Murine but not human mesenchymal stem cells generate osteosarcoma-like lesions in the lung. Stem Cells 2007;25:1586-1594.
[159] Miura M, Miura Y, Padilla-Nash HM, Molinolo AA, Fu B, Patel V*, et al.* Accumulated chromosomal instability in murine bone marrow mesenchymal stem cells leads to malignant transformation. Stem Cells 2006;24:1095-1103.
[160] Tolar J, Nauta AJ, Osborn MJ, Panoskaltsis Mortari A, McElmurry RT, Bell S*, et al.* Sarcoma derived from cultured mesenchymal stem cells. Stem Cells 2007;25:371-379.
[161] Zhou YF, Bosch-Marce M, Okuyama H, Krishnamachary B, Kimura H, Zhang L*, et al.* Spontaneous transformation of cultured mouse bone marrow-derived stromal cells. Cancer Res 2006;66:10849-10854.
[162] Garry DJ, Olson EN. A common progenitor at the heart of development. Cell 2006;127:1101-1104.
[163] Ying QL, Wray J, Nichols J, Batlle-Morera L, Doble B, Woodgett J*, et al.* The ground state of embryonic stem cell self-renewal. Nature 2008;453:519-523.
[164] Zimmermann WH, Melnychenko I, Eschenhagen T. Engineered heart tissue for regeneration of diseased hearts. Biomaterials 2004;25:1639-1647.
[165] Christalla P, Didié M, Eschenhagen T, Tozakidou M, Ehmke H, Field L, Zimmermann WH Fibroblasts are essential for the generation of murine

embryonic stem cell derived bioengeneered myocardium. Abstract ISSCR 7th Annual Meeting. Barcelona: International Society Stem Cell Research, 2009.
[166] Nussbaum J, Minami E, Laflamme MA, Virag JA, Ware CB, Masino A, *et al.* Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response. FASEB J 2007;21:1345-1357.
[167] Grinnemo KH, Genead R, Kumagai-Braesch M, Andersson A, Danielsson C, Mansson-Broberg A, *et al.* Costimulation blockade induces tolerance to HESC transplanted to the testis and induces regulatory T-cells to HESC transplanted into the heart. Stem Cells 2008;26:1850-1857.
[168] He Q, Trindade PT, Stumm M, Li J, Zammaretti P, Bettiol E, *et al.* Fate of undifferentiated mouse embryonic stem cells within the rat heart: role of myocardial infarction and immune suppression. J Cell Mol Med 2009;13:188-201.
[169] Huber I, Itzhaki I, Caspi O, Arbel G, Tzukerman M, Gepstein A, *et al.* Identification and selection of cardiomyocytes during human embryonic stem cell differentiation. FASEB J 2007;21:2551-2563.
[170] Laflamme MA, Chen KY, Naumova AV, Muskheli V, Fugate JA, Dupras SK, *et al.* Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat Biotechnol 2007;25:1015-1024.
[171] Tomescot A, Leschik J, Bellamy V, Dubois G, Messas E, Bruneval P, *et al.* Differentiation in vivo of cardiac committed human embryonic stem cells in postmyocardial infarcted rats. Stem Cells 2007;25:2200-2205.
[172] Behfar A, Perez-Terzic C, Faustino RS, Arrell DK, Hodgson DM, Yamada S, *et al.* Cardiopoietic programming of embryonic stem cells for tumor-free heart repair. J Exp Med 2007;204:405-420.
[173] Oh H, Chi X, Bradfute SB, Mishina Y, Pocius J, Michael LH, *et al.* Cardiac muscle plasticity in adult and embryo by heart-derived progenitor cells. Ann N Y Acad Sci 2004;1015:182-189.
[174] Mouquet F, Pfister O, Jain M, Oikonomopoulos A, Ngoy S, Summer R, *et al.* Restoration of cardiac progenitor cells after myocardial infarction by self-proliferation and selective homing of bone marrow-derived stem cells. Circ Res 2005;97:1090-1092.
[175] Liao R, Pfister O, Jain M, Mouquet F. The bone marrow--cardiac axis of myocardial regeneration. Prog Cardiovasc Dis 2007;50:18-30.
[176] Moretti A, Caron L, Nakano A, Lam JT, Bernshausen A, Chen Y, *et al.* Multipotent embryonic isl1+ progenitor cells lead to cardiac, smooth muscle, and endothelial cell diversification. Cell 2006;127:1151-1165.
[177] Bearzi C, Rota M, Hosoda T, Tillmanns J, Nascimbene A, De Angelis A, *et al.* Human cardiac stem cells. Proc Natl Acad Sci U S A 2007;104:14068-14073.
[178] Smith RR, Barile L, Cho HC, Leppo MK, Hare JM, Messina E, *et al.* Regenerative potential of cardiosphere-derived cells expanded from percutaneous endomyocardial biopsy specimens. Circulation 2007;115:896-908.
[179] Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006;126:663-676.
[180] Okita K, Ichisaka T, Yamanaka S. Generation of germline-competent induced pluripotent stem cells. Nature 2007;448:313-317.

[181] Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K*, et al.* In vitro reprogramming of fibroblasts into a pluripotent ES-cell- like state. Nature 2007;448:318-324.
[182] Maherali N, Sridharan R, Xie W, Utikal J, Eminli S, Arnold K*, et al.* Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. Cell Stem Cell 2007;1:55-70.
[183] Karra R, Wu SM. Multipotent stem cells in cardiac regenerative therapy. Regen Med 2008;3:189-198.
[184] Nelson TJ, Martinez-Fernandez A, Yamada S, Perez-Terzic C, Ikeda Y, Terzic A. Repair of acute myocardial infarction by human stemness factors induced pluripotent stem cells. Circulation 2009;120:408-416.
[185] Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K*, et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131:861-872.
[186] Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S*, et al.* Induced pluripotent stem cell lines derived from human somatic cells. Science 2007;318:1917-1920.
[187] Edel MM, C; Menendez, S; Raya, A; Izpisua Belmonte, JC. The role of the cell cycle in human ESc self-renewal and reprogramming of somatic cells to iPS. Abstract ISSCR 7th Annual Meeting. Barcelona: International Society Stem Cell Research, 2009.
[188] Stamm C, Kleine HD, Choi YH, Dunkelmann S, Lauffs JA, Lorenzen B*, et al.* Intramyocardial delivery of CD133+ bone marrow cells and coronary artery bypass grafting for chronic ischemic heart disease: safety and efficacy studies. J Thorac Cardiovasc Surg 2007;133:717-725.
[189] Yerebakan C, Kaminski A, Liebold A, Steinhoff G. Safety of intramyocardial stem cell therapy for the ischemic myocardium: results of the Rostock trial after 5-year follow-up. Cell Transplant 2007;16:935-940.
[190] Yerebakan C, Kaminski A, Westphal B, Liebold A, Steinhoff G. Autologous bone marrow stem cell therapy for the ischemic myocardium during coronary artery bypass grafting. Minim Invasive Ther Allied Technol 2008;17:143-148.
[191] Bartunek J, Vanderheyden M, Vandekerckhove B, Mansour S, De Bruyne B, De Bondt P*, et al.* Intracoronary injection of CD133-positive enriched bone marrow progenitor cells promotes cardiac recovery after recent myocardial infarction: feasibility and safety. Circulation 2005;112:I178-183.
[192] Ahmadi H, Baharvand H, Ashtiani SK, Soleimani M, Sadeghian H, Ardekani JM*, et al.* Safety analysis and improved cardiac function following local autologous transplantation of CD133(+) enriched bone marrow cells after myocardial infarction. Curr Neurovasc Res 2007;4:153-160.
[193] Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ*, et al.* Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng 2001;7:211-228.
[194] Bieback K, Kern S, Kluter H, Eichler H. Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. Stem Cells 2004;22:625-634.
[195] Kuznetsov SA, Mankani MH, Gronthos S, Satomura K, Bianco P, Robey PG. Circulating skeletal stem cells. J Cell Biol 2001;153:1133-1140.
[196] Jiang Y, Vaessen B, Lenvik T, Blackstad M, Reyes M, Verfaillie CM. Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. Exp Hematol 2002;30:896-904.

[197] Nagaya N, Fujii T, Iwase T, Ohgushi H, Itoh T, Uematsu M, *et al.* Intravenous administration of mesenchymal stem cells improves cardiac function in rats with acute myocardial infarction through angiogenesis and myogenesis. Am J Physiol Heart Circ Physiol 2004;287:H2670-2676.
[198] Dai W, Hale SL, Martin BJ, Kuang JQ, Dow JS, Wold LE, *et al.* Allogeneic mesenchymal stem cell transplantation in postinfarcted rat myocardium: short- and long-term effects. Circulation 2005;112:214-223.
[199] Nesselmann C, Ma N, Bieback K, Wagner W, Ho A, Konttinen YT, *et al.* Mesenchymal stem cells and cardiac repair. J Cell Mol Med 2008;12:1795-1810.
[200] Chen SL, Fang WW, Ye F, Liu YH, Qian J, Shan SJ, *et al.* Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. Am J Cardiol 2004;94:92-95.
[201] Burt RK, Loh Y, Pearce W, Beohar N, Barr WG, Craig R, *et al.* Clinical applications of blood-derived and marrow-derived stem cells for nonmalignant diseases. JAMA 2008;299:925-936.
[202] Uemura R, Xu M, Ahmad N, Ashraf M. Bone marrow stem cells prevent left ventricular remodeling of ischemic heart through paracrine signaling. Circ Res 2006;98:1414-1421.
[203] Gnecchi M, Zhang Z, Ni A, Dzau VJ. Paracrine mechanisms in adult stem cell signaling and therapy. Circ Res 2008;103:1204-1219.
[204] Psaltis PJ, Zannettino AC, Worthley SG, Gronthos S. Concise review: mesenchymal stromal cells: potential for cardiovascular repair. Stem Cells 2008;26:2201-2210.
[205] Quevedo HC, Hatzistergos KE, Oskouei BN, Feigenbaum GS, Rodriguez JE, Valdes D, *et al.* Allogeneic mesenchymal stem cells restore cardiac function in chronic ischemic cardiomyopathy via trilineage differentiating capacity. Proc Natl Acad Sci U S A 2009.
[206] Barcelos LS, Duplaa C, Krankel N, Graiani G, Invernici G, Katare R, *et al.* Human CD133+ progenitor cells promote the healing of diabetic ischemic ulcers by paracrine stimulation of angiogenesis and activation of Wnt signaling. Circ Res 2009;104:1095-1102.
[207] Chang MG, Tung L, Sekar RB, Chang CY, Cysyk J, Dong P, *et al.* Proarrhythmic potential of mesenchymal stem cell transplantation revealed in an in vitro coculture model. Circulation 2006;113:1832-1841.
[208] Davis ME, Hsieh PC, Takahashi T, Song Q, Zhang S, Kamm RD, *et al.* Local myocardial insulin-like growth factor 1 (IGF-1) delivery with biotinylated peptide nanofibers improves cell therapy for myocardial infarction. Proc Natl Acad Sci U S A 2006;103:8155-8160.
[209] Askari AT, Unzek S, Popovic ZB, Goldman CK, Forudi F, Kiedrowski M, *et al.* Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. Lancet 2003;362:697-703.
[210] Kaminski A, Ma N, Donndorf P, Lindenblatt N, Feldmeier G, Ong LL, *et al.* Endothelial NOS is required for SDF-1alpha/CXCR4-mediated peripheral endothelial adhesion of c-kit+ bone marrow stem cells. Lab Invest 2008;88:58-69.
[211] Fox JM, Chamberlain G, Ashton BA, Middleton J. Recent advances into the understanding of mesenchymal stem cell trafficking. Br J Haematol 2007;137:491-502.

[212] Gabrilove J. The development of granulocyte colony-stimulating factor in its various clinical applications. Blood 1992;80:1382-1385.
[213] Lieschke GJ, Maher D, O'Connor M, Green M, Sheridan W, Rallings M, *et al.* Phase I study of intravenously administered bacterially synthesized granulocyte-macrophage colony-stimulating factor and comparison with subcutaneous administration. Cancer Res 1990;50:606-614.
[214] Lieschke GJ, Burgess AW. Granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor (1). N Engl J Med 1992;327:28-35.
[215] Anderlini P, Przepiorka D, Champlin R, Korbling M. Biologic and clinical effects of granulocyte colony-stimulating factor in normal individuals. Blood 1996;88:2819-2825.
[216] Falanga A, Marchetti M, Evangelista V, Manarini S, Oldani E, Giovanelli S, *et al.* Neutrophil activation and hemostatic changes in healthy donors receiving granulocyte colony-stimulating factor. Blood 1999;93:2506-2514.
[217] Minatoguchi S, Takemura G, Chen XH, Wang N, Uno Y, Koda M, *et al.* Acceleration of the healing process and myocardial regeneration may be important as a mechanism of improvement of cardiac function and remodeling by postinfarction granulocyte colony-stimulating factor treatment. Circulation 2004;109:2572-2580.
[218] Ohtsuka M, Takano H, Zou Y, Toko H, Akazawa H, Qin Y, *et al.* Cytokine therapy prevents left ventricular remodeling and dysfunction after myocardial infarction through neovascularization. FASEB J 2004;18:851-853.
[219] Kanellakis P, Slater NJ, Du XJ, Bobik A, Curtis DJ. Granulocyte colony-stimulating factor and stem cell factor improve endogenous repair after myocardial infarction. Cardiovasc Res 2006;70:117-125.
[220] Ripa RS, Jorgensen E, Wang Y, Thune JJ, Nilsson JC, Sondergaard L, *et al.* Stem cell mobilization induced by subcutaneous granulocyte-colony stimulating factor to improve cardiac regeneration after acute ST-elevation myocardial infarction: result of the double-blind, randomized, placebo-controlled stem cells in myocardial infarction (STEMMI) trial. Circulation 2006;113:1983-1992.
[221] Abdel-Latif A, Bolli R, Zuba-Surma EK, Tleyjeh IM, Hornung CA, Dawn B. Granulocyte colony-stimulating factor therapy for cardiac repair after acute myocardial infarction: a systematic review and meta-analysis of randomized controlled trials. Am Heart J 2008;156:216-226 e219.
[222] Jelkmann W, Wagner K. Beneficial and ominous aspects of the pleiotropic action of erythropoietin. Ann Hematol 2004;83:673-686.
[223] Zwezdaryk KJ, Coffelt SB, Figueroa YG, Liu J, Phinney DG, LaMarca HL, *et al.* Erythropoietin, a hypoxia-regulated factor, elicits a pro-angiogenic program in human mesenchymal stem cells. Exp Hematol 2007;35:640-652.
[224] Calvillo L, Latini R, Kajstura J, Leri A, Anversa P, Ghezzi P, *et al.* Recombinant human erythropoietin protects the myocardium from ischemia-reperfusion injury and promotes beneficial remodeling. Proc Natl Acad Sci U S A 2003;100:4802-4806.
[225] Parsa CJ, Matsumoto A, Kim J, Riel RU, Pascal LS, Walton GB, *et al.* A novel protective effect of erythropoietin in the infarcted heart. J Clin Invest 2003;112:999-1007.
[226] Tramontano AF, Muniyappa R, Black AD, Blendea MC, Cohen I, Deng L, *et al.* Erythropoietin protects cardiac myocytes from hypoxia-induced apoptosis

through an Akt-dependent pathway. Biochem Biophys Res Commun 2003;308:990-994.
[227] Vandervelde S, van Luyn MJ, Tio RA, Harmsen MC. Signaling factors in stem cell-mediated repair of infarcted myocardium. J Mol Cell Cardiol 2005;39:363-376.
[228] Moon C, Krawczyk M, Ahn D, Ahmet I, Paik D, Lakatta EG, *et al.* Erythropoietin reduces myocardial infarction and left ventricular functional decline after coronary artery ligation in rats. Proc Natl Acad Sci U S A 2003;100:11612-11617.
[229] Asaumi Y, Kagaya Y, Takeda M, Yamaguchi N, Tada H, Ito K, *et al.* Protective role of endogenous erythropoietin system in nonhematopoietic cells against pressure overload-induced left ventricular dysfunction in mice. Circulation 2007;115:2022-2032.
[230] Cai Z, Manalo DJ, Wei G, Rodriguez ER, Fox-Talbot K, Lu H, *et al.* Hearts from rodents exposed to intermittent hypoxia or erythropoietin are protected against ischemia-reperfusion injury. Circulation 2003;108:79-85.
[231] Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, *et al.* Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 1999;85:221-228.
[232] Kalka C, Masuda H, Takahashi T, Kalka-Moll WM, Silver M, Kearney M, *et al.* Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proc Natl Acad Sci U S A 2000;97:3422-3427.
[233] Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, *et al.* Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med 1999;5:434-438.
[234] Kawamoto A, Gwon HC, Iwaguro H, Yamaguchi JI, Uchida S, Masuda H, *et al.* Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. Circulation 2001;103:634-637.
[235] Heeschen C, Aicher A, Lehmann R, Fichtlscherer S, Vasa M, Urbich C, *et al.* Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. Blood 2003;102:1340-1346.
[236] Bahlmann FH, DeGroot K, Duckert T, Niemczyk E, Bahlmann E, Boehm SM, *et al.* Endothelial progenitor cell proliferation and differentiation is regulated by erythropoietin. Kidney Int 2003;64:1648-1652.
[237] Bahlmann FH, De Groot K, Spandau JM, Landry AL, Hertel B, Duckert T, *et al.* Erythropoietin regulates endothelial progenitor cells. Blood 2004;103:921-926.
[238] George J, Goldstein E, Abashidze A, Wexler D, Hamed S, Shmilovich H, *et al.* Erythropoietin promotes endothelial progenitor cell proliferative and adhesive properties in a PI 3-kinase-dependent manner. Cardiovasc Res 2005;68:299-306.
[239] Lindahl B, Toss H, Siegbahn A, Venge P, Wallentin L. Markers of myocardial damage and inflammation in relation to long-term mortality in unstable coronary artery disease. FRISC Study Group. Fragmin during Instability in Coronary Artery Disease. N Engl J Med 2000;343:1139-1147.
[240] Moller JE, Hillis GS, Oh JK, Pellikka PA. Prognostic importance of secondary pulmonary hypertension after acute myocardial infarction. Am J Cardiol 2005;96:199-203.
[241] Gao E, Boucher M, Chuprun JK, Zhou RH, Eckhart AD, Koch WJ. Darbepoetin alfa, a long-acting erythropoietin analog, offers novel and delayed

cardioprotection for the ischemic heart. Am J Physiol Heart Circ Physiol 2007;293:H60-68.
[242] Prunier F, Pfister O, Hadri L, Liang L, Del Monte F, Liao R, *et al.* Delayed erythropoietin therapy reduces post-MI cardiac remodeling only at a dose that mobilizes endothelial progenitor cells. Am J Physiol Heart Circ Physiol 2007;292:H522-529.
[243] Besarab A, Bolton WK, Browne JK, Egrie JC, Nissenson AR, Okamoto DM, *et al.* The effects of normal as compared with low hematocrit values in patients with cardiac disease who are receiving hemodialysis and epoetin. N Engl J Med 1998;339:584-590.
[244] van der Meer P, Voors AA, Lipsic E, Smilde TD, van Gilst WH, van Veldhuisen DJ. Prognostic value of plasma erythropoietin on mortality in patients with chronic heart failure. J Am Coll Cardiol 2004;44:63-67.
[245] Shingo T, Sorokan ST, Shimazaki T, Weiss S. Erythropoietin regulates the in vitro and in vivo production of neuronal progenitors by mammalian forebrain neural stem cells. J Neurosci 2001;21:9733-9743.
[246] Burger D, Lei M, Geoghegan-Morphet N, Lu X, Xenocostas A, Feng Q. Erythropoietin protects cardiomyocytes from apoptosis via up-regulation of endothelial nitric oxide synthase. Cardiovasc Res 2006;72:51-59.
[247] Feng Q. Beyond erythropoiesis: the anti- inflammatory effects of erythropoietin. Cardiovasc Res 2006;71:615-617.
[248] Hirata A, Minamino T, Asanuma H, Fujita M, Wakeno M, Myoishi M, *et al.* Erythropoietin enhances neovascularization of ischemic myocardium and improves left ventricular dysfunction after myocardial infarction in dogs. J Am Coll Cardiol 2006;48:176-184.
[249] Gazitt Y. Homing and mobilization of hematopoietic stem cells and hematopoietic cancer cells are mirror image processes, utilizing similar signaling pathways and occurring concurrently: circulating cancer cells constitute an ideal target for concurrent treatment with chemotherapy and antilineage-specific antibodies. Leukemia 2004;18:1-10.
[250] Lataillade JJ, Clay D, Dupuy C, Rigal S, Jasmin C, Bourin P, *et al.* Chemokine SDF-1 enhances circulating CD34(+) cell proliferation in synergy with cytokines: possible role in progenitor survival. Blood 2000;95:756-768.
[251] Wright DE, Bowman EP, Wagers AJ, Butcher EC, Weissman IL. Hematopoietic stem cells are uniquely selective in their migratory response to chemokines. J Exp Med 2002;195:1145-1154.
[252] Ponomaryov T, Peled A, Petit I, Taichman RS, Habler L, Sandbank J, *et al.* Induction of the chemokine stromal-derived factor-1 following DNA damage improves human stem cell function. J Clin Invest 2000;106:1331-1339.
[253] McGrath KE, Koniski AD, Maltby KM, McGann JK, Palis J. Embryonic expression and function of the chemokine SDF-1 and its receptor, CXCR4. Dev Biol 1999;213:442-456.
[254] Aiuti A, Webb IJ, Bleul C, Springer T, Gutierrez-Ramos JC. The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. J Exp Med 1997;185:111-120.
[255] Jo DY, Rafii S, Hamada T, Moore MA. Chemotaxis of primitive hematopoietic cells in response to stromal cell-derived factor-1. J Clin Invest 2000;105:101-111.

[256] Wang W, Li W, Ong LL, Furlani D, Kaminski A, Liebold A, *et al.* Localized SDF-1alpha gene release mediated by collagen substrate induces CD117+ stem cell homing. J Cell Mol Med 2008.
[257] Petit I, Szyper-Kravitz M, Nagler A, Lahav M, Peled A, Habler L, *et al.* G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. Nat Immunol 2002;3:687-694.
[258] Mohle R, Bautz F, Rafii S, Moore MA, Brugger W, Kanz L. The chemokine receptor CXCR-4 is expressed on CD34+ hematopoietic progenitors and leukemic cells and mediates transendothelial migration induced by stromal cell-derived factor-1. Blood 1998;91:4523-4530.
[259] Murdoch C. CXCR4: chemokine receptor extraordinaire. Immunol Rev 2000;177:175-184.
[260] Focosi D, Kast RE, Galimberti S, Petrini M. Conditioning response to granulocyte colony-stimulating factor via the dipeptidyl peptidase IV-adenosine deaminase complex. J Leukoc Biol 2008;84:331-337.
[261] Valenzuela-Fernandez A, Planchenault T, Baleux F, Staropoli I, Le-Barillec K, Leduc D, *et al.* Leukocyte elastase negatively regulates Stromal cell-derived factor-1 (SDF-1)/CXCR4 binding and functions by amino-terminal processing of SDF-1 and CXCR4. J Biol Chem 2002;277:15677-15689.
[262] Levesque JP, Hendy J, Takamatsu Y, Williams B, Winkler IG, Simmons PJ. Mobilization by either cyclophosphamide or granulocyte colony-stimulating factor transforms the bone marrow into a highly proteolytic environment. Exp Hematol 2002;30:440-449.
[263] Levesque JP, Hendy J, Takamatsu Y, Simmons PJ, Bendall LJ. Disruption of the CXCR4/CXCL12 chemotactic interaction during hematopoietic stem cell mobilization induced by GCSF or cyclophosphamide. J Clin Invest 2003;111:187-196.
[264] van Os R, van Schie ML, Willemze R, Fibbe WE. Proteolytic enzyme levels are increased during granulocyte colony-stimulating factor-induced hematopoietic stem cell mobilization in human donors but do not predict the number of mobilized stem cells. J Hematother Stem Cell Res 2002;11:513-521.
[265] Levesque JP, Liu F, Simmons PJ, Betsuyaku T, Senior RM, Pham C, *et al.* Characterization of hematopoietic progenitor mobilization in protease-deficient mice. Blood 2004;104:65-72.
[266] Ruster B, Gottig S, Ludwig RJ, Bistrian R, Muller S, Seifried E, *et al.* Mesenchymal stem cells display coordinated rolling and adhesion behavior on endothelial cells. Blood 2006;108:3938-3944.
[267] Chandrasekharan UM, Siemionow M, Unsal M, Yang L, Poptic E, Bohn J, *et al.* Tumor necrosis factor alpha (TNF-alpha) receptor-II is required for TNF-alpha-induced leukocyte-endothelial interaction in vivo. Blood 2007;109:1938-1944.
[268] Sumagin R, Sarelius IH. TNF-alpha activation of arterioles and venules alters distribution and levels of ICAM-1 and affects leukocyte-endothelial cell interactions. Am J Physiol Heart Circ Physiol 2006;291:H2116-2125.
[269] Dercksen MW, Gerritsen WR, Rodenhuis S, Dirkson MK, Slaper-Cortenbach IC, Schaasberg WP, *et al.* Expression of adhesion molecules on CD34+ cells: CD34+ L-selectin+ cells predict a rapid platelet recovery after peripheral blood stem cell transplantation. Blood 1995;85:3313-3319.
[270] Kroger N, Zeller W, Hassan HT, Dierlamm J, Zander AR. Difference between expression of adhesion molecules on CD34+ cells from bone marrow and G-CSF-stimulated peripheral blood. Stem Cells 1998;16:49-53.

[271] Voermans C, Rood PM, Hordijk PL, Gerritsen WR, van der Schoot CE. Adhesion molecules involved in transendothelial migration of human hematopoietic progenitor cells. Stem Cells 2000;18:435-443.
[272] Kronenwett R, Martin S, Haas R. The role of cytokines and adhesion molecules for mobilization of peripheral blood stem cells. Stem Cells 2000;18:320-330.
[273] Williams DA, Rios M, Stephens C, Patel VP. Fibronectin and VLA-4 in haematopoietic stem cell-microenvironment interactions. Nature 1991;352:438-441.
[274] Simmons PJ, Levesque JP, Zannettino AC. Adhesion molecules in haemopoiesis. Baillieres Clin Haematol 1997;10:485-505.
[275] Prosper F, Stroncek D, McCarthy JB, Verfaillie CM. Mobilization and homing of peripheral blood progenitors is related to reversible downregulation of alpha4 beta1 integrin expression and function. J Clin Invest 1998;101:2456-2467.
[276] Bellucci R, De Propris MS, Buccisano F, Lisci A, Leone G, Tabilio A, *et al.* Modulation of VLA-4 and L- selectin expression on normal CD34+ cells during mobilization with G-CSF. Bone Marrow Transplant 1999;23:1-8.
[277] Peled A, Kollet O, Ponomaryov T, Petit I, Franitza S, Grabovsky V, *et al.* The chemokine SDF-1 activates the integrins LFA-1, VLA-4, and VLA-5 on immature human CD34(+) cells: role in transendothelial/stromal migration and engraftment of NOD/SCID mice. Blood 2000;95:3289-3296.
[278] Liu ZJ, Velazquez OC. Hyperoxia, endothelial progenitor cell mobilization, and diabetic wound healing. Antioxid Redox Signal 2008;10:1869-1882.
[279] Aicher A, Heeschen C, Mildner-Rihm C, Urbich C, Ihling C, Technau-Ihling K, *et al.* Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. Nat Med 2003;9:1370-1376.
[280] Heissig B, Hattori K, Dias S, Friedrich M, Ferris B, Hackett NR, *et al.* Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. Cell 2002;109:625-637.
[281] Heissig B, Werb Z, Rafii S, Hattori K. Role of c-kit/Kit ligand signaling in regulating vasculogenesis. Thromb Haemost 2003;90:570-576.
[282] Aicher A, Zeiher AM, Dimmeler S. Mobilizing endothelial progenitor cells. Hypertension 2005;45:321-325.
[283] Kashii Y, Uchida M, Kirito K, Tanaka M, Nishijima K, Toshima M, *et al.* A member of Forkhead family transcription factor, FKHRL1, is one of the downstream molecules of phosphatidylinositol 3-kinase-Akt activation pathway in erythropoietin signal transduction. Blood 2000;96:941-949.
[284] Ghezzi P, Brines M. Erythropoietin as an antiapoptotic, tissue-protective cytokine. Cell Death Differ 2004;11 Suppl 1:S37-44.
[285] Buemi M, Lacquaniti A, Maricchiolo G, Bolignano D, Campo S, Cernaro V, *et al.* Regenerative medicine: does Erythropoietin have a role? Curr Pharm Des 2009;15:2026-2036.
[286] Peled A, Petit I, Kollet O, Magid M, Ponomaryov T, Byk T, *et al.* Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. Science 1999;283:845-848.
[287] Behr L, Hekmati M, Fromont G, Borenstein N, Noel LH, Lelievre-Pegorier M, *et al.* Intra renal arterial injection of autologous mesenchymal stem cells in an ovine model in the postischemic kidney. Nephron Physiol 2007;107:p65-76.
[288] Walczak P, Zhang J, Gilad AA, Kedziorek DA, Ruiz-Cabello J, Young RG, *et al.* Dual-modality monitoring of targeted intraarterial delivery of mesenchymal stem cells after transient ischemia. Stroke 2008;39:1569-1574.

[289] Bartsch T, Brehm M, Zeus T, Kogler G, Wernet P, Strauer BE. Transplantation of autologous mononuclear bone marrow stem cells in patients with peripheral arterial disease (the TAM-PAD study). Clin Res Cardiol 2007;96:891-899.
[290] Freyman T, Polin G, Osman H, Crary J, Lu M, Cheng L, *et al.* A quantitative, randomized study evaluating three methods of mesenchymal stem cell delivery following myocardial infarction. Eur Heart J 2006;27:1114-1122.
[291] Vulliet PR, Greeley M, Halloran SM, MacDonald KA, Kittleson MD. Intra-coronary arterial injection of mesenchymal stromal cells and microinfarction in dogs. Lancet 2004;363:783-784.
[292] Schmidt RF. GT, eds.Second Edition. ed. New York: Springer-Verlag; . 1989.
[293] Chien S. iDMS. Biophysical Behavior of Red Cells in Suspensions. 2nd Edition ed. NY: Academic Press; . 1975.
[294] Lipowsky HH. CBM, Junji Seki. Trans Time Distributions of Blood Flow in the Microcirculation. NY: Springer-Verlag; . 1989.
[295] Guyton AC. JEH. Human Physiology and Mechanisms of Disease. Sixth Edition ed. Philadelphia PA: W.B. Saunders Company; . 1997.
[296] Gaehtgens P. Flow of blood through narrow capillaries: Rheological mechanisms determining capillary hematocrit and apparent viscosity; . 1980.
[297] Barbash IM, Chouraqui P, Baron J, Feinberg MS, Etzion S, Tessone A, *et al.* Systemic delivery of bone marrow-derived mesenchymal stem cells to the infarcted myocardium: feasibility, cell migration, and body distribution. Circulation 2003;108:863-868.
[298] Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, *et al.* Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. Nat Med 2001;7:430-436.
[299] Pittenger MF, Martin BJ. Mesenchymal stem cells and their potential as cardiac therapeutics. Circ Res 2004;95:9-20.
[300] Aicher A, Brenner W, Zuhayra M, Badorff C, Massoudi S, Assmus B, *et al.* Assessment of the tissue distribution of transplanted human endothelial progenitor cells by radioactive labeling. Circulation 2003;107:2134-2139.
[301] Hofmann M, Wollert KC, Meyer GP, Menke A, Arseniev L, Hertenstein B, *et al.* Monitoring of bone marrow cell homing into the infarcted human myocardium. Circulation 2005;111:2198-2202.
[302] Bel A, Messas E, Agbulut O, Richard P, Samuel JL, Bruneval P, *et al.* Transplantation of autologous fresh bone marrow into infarcted myocardium: a word of caution. Circulation 2003;108 Suppl 1:II247-252.
[303] Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature 2001;414:105-111.
[304] Reya T, Clevers H. Wnt signalling in stem cells and cancer. Nature 2005;434:843-850.
[305] Penna C, Raimondo S, Ronchi G, Rastaldo R, Mancardi D, Cappello S, *et al.* Early homing of adult mesenchymal stem cells in normal and infarcted isolated beating hearts. J Cell Mol Med 2008;12:507-521.
[306] Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, *et al.* Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 2002;418:41-49.
[307] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, *et al.* Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143-147.

[308] Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, Fike JR, Lee HO, Pfeffer K, *et al.* Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. Nature 2003;425:968-973.
[309] Vassilopoulos G, Wang PR, Russell DW. Transplanted bone marrow regenerates liver by cell fusion. Nature 2003;422:901-904.
[310] Wang X, Willenbring H, Akkari Y, Torimaru Y, Foster M, Al-Dhalimy M, *et al.* Cell fusion is the principal source of bone-marrow-derived hepatocytes. Nature 2003;422:897-901.
[311] Spees JL, Olson SD, Ylostalo J, Lynch PJ, Smith J, Perry A, *et al.* Differentiation, cell fusion, and nuclear fusion during ex vivo repair of epithelium by human adult stem cells from bone marrow stroma. Proc Natl Acad Sci U S A 2003;100:2397-2402.
[312] Li H, Fan X, Kovi RC, Jo Y, Moquin B, Konz R, *et al.* Spontaneous expression of embryonic factors and p53 point mutations in aged mesenchymal stem cells: a model of age-related tumorigenesis in mice. Cancer Res 2007;67:10889-10898.
[313] Prockop DJ, Olson SD. Clinical trials with adult stem/progenitor cells for tissue repair: let's not overlook some essential precautions. Blood 2007;109:3147-3151.
[314] Wang Y, Huso DL, Harrington J, Kellner J, Jeong DK, Turney J, *et al.* Outgrowth of a transformed cell population derived from normal human BM mesenchymal stem cell culture. Cytotherapy 2005;7:509-519.

10. Financial support

This work was supported by the German Helmholtz Association, Mecklenburg-Vorpommern (Nachwuchsgruppe Regenerative Medizin Regulation der Stammzellmigration 0402710), German Research Foundation, Sonderforschungsbereich / Transregio 37, B5, B2 and A4, START-MSC (project 6: Kardiovaskuläre Differenzierung und Applikation definierter mesenchymaler Stammzellpopulationen), Miltenyi Biotec and Referenz- und Translationszentrum für kardiale Stammzelltherapien (RTC) der Universität Rostock. DAAD Project Based Personnel Exchange Programme with China.

11. Publications list

1:

Furlani D, Li W, Pittermann E, Klopsch C, Wang L, Knopp A, Jungebluth P, Thedinga E, Havenstein C, Westien I, Ugurlucan M, Li RK, Ma N, Steinhoff G. A transformed cell population derived from cultured mesenchymal stem cells has no functional effect after transplantation into the injured heart. *Cell Transplant*. 2009;18(3):319-31. PMID: 19558780 [PubMed - indexed for MEDLINE].

2:

Yerebakan C, Sandica E, Prietz S, Klopsch C, Ugurlucan M, Kaminski A, Abdija S, Lorenzen B, Boltze J, Nitzsche B, Egger D, Barten M, **Furlani D**, Ma N, Vollmar B, Liebold A, Steinhoff G. Autologous umbilical cord blood mononuclear cell transplantation preserves right ventricular function in a novel model of chronic right ventricular volume overload. *Cell Transplant*. 2009 Apr 9. pii: CT-2015. [Epub ahead of print] PMID: 19500473 [PubMed - as supplied by publisher].

3:

Klopsch C*, **Furlani D***, Gäbel R, Li W, Pittermann E, Ugurlucan M, Kundt G, Zingler C, Titze U, Wang W, Ong LL, Wagner K, Li RK, Ma N, Steinhoff G. Intracardiac injection of erythropoietin induces stem cell recruitment and improves cardiac functions in a rat myocardial infarction model. *J Cell Mol Med*. 2009;13(4):664-79. PMID: 19449462 [PubMed - indexed for MEDLINE.*Equally contributed.

4:

Wang W, Li W, Ong LL, Lutzow K, Lendlein A, **Furlani D**, Gabel R, Kong D, Wang J, Li RK, Steinhoff G, Ma N. Localized and sustained SDF-1 gene release mediated by fibronectin films: A potential method for recruiting stem cells. *Int J Artif Organs*. 2009;32(3):141-9. PMID: 19440989 [PubMed - in process].

5:

Wang W, Li W, Ong LL, **Furlani D**, Kaminski A, Liebold A, Lützow K, Lendlein A, Wang J, Li RK, Steinhoff G, Ma N. Localized SDF-1alpha gene release mediated by collagen substrate induces CD117+ stem cell homing. *J Cell Mol Med*. 2008 Dec 24. [Epub ahead of print] PMID: 19413887 [PubMed - as supplied by publisher].

6:

Gäbel R, Klopsch C, **Furlani D**, Yerebakan C, Li W, Ugurlucan M, Ma N, Steinhoff G. Single high-dose intramyocardial administration of erythropoietin promotes early intracardiac proliferation, proves safety and restores cardiac performance after myocardial infarction in rats. *Interact Cardiovasc Thorac Surg*. 2009;9(1):20-5; discussion 25. Epub 2009 Apr 20. PMID: 19380336 [PubMed - in process].

7:

Furlani D, Francesco Ficetola G, Colombo G, Ugurlucan M, De Bernardi F. Deforestation and the structure of frog communities in the Humedale Terraba-Sierpe, Costa Rica. *Zoolog Sci*. 2009;26(3):197-202. PMID: 19341340 [PubMed - in process].

8:

Furlani D, Ugurlucan M, Ong L, Bieback K, Pittermann E, Westien I, Wang W, Yerebakan C, Li W, Gäbel R, Li RK, Vollmar B, Steinhoff G, Ma N. Is the intravascular administration of mesenchymal stem cells safe? Mesenchymal stem cells and intravital microscopy. *Microvasc Res*. 2009;77(3):370-6. Epub 2009 Feb 26. PMID: 19249320 [PubMed - indexed for MEDLINE].

9:

Ugurlucan M, Yerebakan C, **Furlani D**, Ma N, Steinhoff G. Cell sources for cardiovascular tissue regeneration and engineering. *Thorac Cardiovasc Surg*. 2009;57(2):63-73. Epub 2009 Feb 24. Review. PMID: 19241306 [PubMed - indexed for MEDLINE].

10:

Ugurlucan M, **Furlani D**, Klopsch C, Steinhoff G. eComment: left ventricular catheterization for pressure-volume loop measurements in small laboratory animals. *Interact Cardiovasc Thorac Surg*. 2008;7(5):927; discussion 927. No abstract available. PMID: 18801819 [PubMed - indexed for MEDLINE].

11:

Furlani D, Klopsch C, Gäbel R, Ugurlucan M, Pittermann E, Klee D, Wagner K, Li W, Wang W, Ong LL, Nizze H, Titze U, Lützow K, Lendlein A, Steinhoff G, Ma N. Intracardiac erythropoietin injection reveals antiinflammatory potential and improved cardiac functions detected by Forced Swim Test. *Transplant Proc*. 2008;40(4):962-6. PMID: 18555090 [PubMed - indexed for MEDLINE].

12:

Kaminski A, Ma N, Donndorf P, Lindenblatt N, Feldmeier G, Ong LL, **Furlani D**, Skrabal CA, Liebold A, Vollmar B, Steinhoff G. Endothelial NOS is required for SDF-1alpha/CXCR4-mediated peripheral endothelial adhesion of c-kit + bone marrow stem cells. *Lab Invest*. 2008;88(1):58-69. Epub 2007 Nov 26. PMID: 18040270 [PubMed - indexed for MEDLINE].

13:

Ficetola, G.F., **Furlani, D**., Colombo, G., De Bernardi, F. Assessing the value of secondary forest for Amphibians: *Eleutherodactylus* frogs in a gradient of forest alteration. *Biodiversity and Conservation*. 2008 17, 2185-2195.

14:

Li W, Ma N, Ong LL, Nesselmann C, Klopsch C, Ladilov Y, **Furlani D**, Piechaczek C, Moebius JM, Lützow K, Lendlein A, Stamm C, Li RK, Steinhoff G. Bcl-2 engineered MSCs inhibited apoptosis and improved heart function. Stem Cells. 2007;25(8):2118-27. Epub 2007 May 3. PMID: 17478584 [PubMed - indexed for MEDLINE].

12. Abstract presentations and conferences

1:

Furlani D, Gäbel R, Ong LL, Li W, Steinhoff G, Ma N. High-mobility group protein box 1 (HMGB1) induces cytoskeleton reorganization in mouse bone marrow c-Kit+ cells. Annual meeting of German Society for Stem Cell Research, November 2006. Köln, Germany.

2:

Ma N, Li W, **Furlani D**, Ong LL, Luetzow K, Klopsch C, Liebold A, Lendlein A, Steinhoff G. A transformed cell population derived from long term cultured mesenchymal stem cells has no functional effect after transplantation into the injured heart. Annual meeting of the American Society of Gene Therapy, May 30 – June 3, 2007. Seattle, Washington, USA.

3:

Klopsch C, **Furlani D**, Gäbel R, Wagner K, Wang W, Ong LL, Li W, Nizze H, Titze U, Lendlein A, Lützow K, Li RK, Ma N, Steinhoff G. Intracardiac injection of Epoetin-□ upregulates stem cell chemoattractant gene expression in a rat myocardial infarction model. Rostocker Gespräche über kardiovaskuläre Funktion und Hypertonie, June 9, 2007. Rostock, Germany.

4:

Furlani D, Klopsch C, Gäbel R, Wagner K, Li W, Ugurlucan M, Wang W, Ong LL, Nizze H, Titze U, Ma N, Steinhoff G. Intracardiac Injection of Epoetin-a Upregulates Stem Cell Chemoattractant Gene Expression Promoting Myocardial Regeneration in a Rodent Ischemia Model. Annual meeting of German Society for Stem Cell Research, October, 2007. Würzburg, Germany.

5:

Furlani D, Klopsch C, Gäbel R, Wagner K, Ugurlucan M, Wang W, Ong LL, Li W, Nizze H, Titze U, Liebold A, Ma N, Steinhoff G. Intracardiac Injection of Epoetin-a Upregulates SDF-1 Gene Expression Promoting Myocardial Regeneration in a Rat Myocardial Infarction. Konferenz Regenerative Medizin Mikro und Nanotechnologien im Bereich der Regenerativen Medizin, October 10, 2007. Hannover, Germany.

6:

Klopsch C, **Furlani D**, Gäbel R, Wagner K, Wang W, Ong LL, Li W, Nizze H, Titze U, Peuster M, Steinhoff G, Ma N. Intramyokardiale Injektion von Epoetin-α beeinflusst den Heilungsprozess nach akutem Herzinfarkt in Ratten, verbessert Herzfunktion und verringert pulmonale Hypertension. 16. Jahrestagung der Deutschen TransplantationsGesellschaft, October 11 – 13, 2007. Mainz, Germany.

7:

Furlani D, Klopsch C, Gäbel R, Wagner K, Wang W, Ong LL, Li W, Nizze H, Titze U, Lendlein A, Lützow K, Li R, Ma N, and Steinhoff G. Intracardiac Injection of Epoetin-Upregulates Stem Cell Chemoattractant Gene Expression In a Rat Myocardial Infarction Model.

Circulation, Oct 2007; 116: II_212 - II_213. Annual meeting of the American Heart Association, Scientific Sessions, November 5 – 8, 2007. Orlando, Florida, USA.

8:

Furlani D, Klopsch C, Gäbel R, Wagner K, Ugurlucan M, Li W, Wang W, Ong LL, Liebold A, Ma N, Steinhoff G. Intracardiac injection of epoetin upregulates SDF-1 gene expression promoting myocardial regeneration in a rat myocardium infarction. 5 Gemeinsamen Jahrestagung der Deutschen, Österreichischen und Schweizer Gesellschaften für Thorax-, Herz- und Gefässchirurgie (D-A-CH), February 17 – 20, 2008. Insbruck, Austria.

9:

Furlani D , Ugurlucan M, Li W, Ma N, Jungebluth P, Ong LL, Pittermann E, Klopsch C, Luetzow K, Lendlein A, Steinhoff G. Transformed Cells Appeared in Early Passage Cultured Mesenchymal Stem Cells Have No Beneficial Effect after Transplantation into the Injured Heart. *Molecular Therapy* Volume 16, Supplement 1, May 2008. Annual meeting of the American Society of Gene Therapy, May 28 – June 1, 2008. Boston, Massachusetts, USA.

10:

Ugurlucan M, **Furlani D**, Pittermann E, Ong LL, Yerebakan C, Gäbel R, Klopsch C, Wang W, Li W, Ma N, Steinhoff G. Is Intravascular Transplantation of Mesenchymal Stem Cells Safe? *Molecular Therapy* Volume 16, Supplement 1, May 2008. Annual meeting of the American Society of Gene Therapy, May 28 – June 1, 2008. Boston, Massachusetts, USA.

11:

Furlani D , Ugurlucan M, Ong LL, Wang W, Pittermann E, Kaminski A, Donndorf P, Yerebakan C, Ma N, Vollmar B, Steinhoff G. Mesenchymal Stem Cells Following Intraarterial Injection: an Intravital Microscopy Study in a Rodent Model. 2nd International Congress on Stem Cells and Tissue Formation, May 28 – June 1, 2008. Dresden, Germany.

12:

Klopsch C, **Furlani D** , Gäbel R, Wagner K, Wang W, Ong LL, Li W, Nizze H, Titze U, Lendlein A, Lützow K, Li RK, Ma N, Steinhoff G. Intrakardiale Injektion von Epoetin induziert Hochregulierung von Genen chemoattraktiver Moleküle zur Rekrutierung von Stammzellen nach akutem Myokardinfarkt in Ratten. 37. Rostocker Gespräche über kardiovaskuläre Funktion und Hypertonie, 31. Mai 2008, Rostock, Germany.

13:

Wang W, Li W, Ong LL, **Furlani D**, Gäbel R, Ugurlucan M, Klopsch C, Lorenz P, Lützow K, Lendlein A, Ma N, Steinhoff G. Collagen and fibronectin substrates mediated local SDF-1 gene expression induces stem cells homing. European Society for Artificial Organs (ESAO): Annual Meeting, 3 – 6 September 2008, Centre Médical Universitaire, Geneva, Switzerland.

14:

Gäbel R, Klopsch C, **Furlani D** , Li W, Ugurlucan M, Ong LL, Ma N, Steinhoff G. Intramyokardiale Erythropoietin-Injektion vermittelt die Rekrutierung von Stammzellen und regeneriert kardiale Funktionen nach Myokardinfarkt im Rattenmodell. 22nd The European

Association for Cardio – Thoracic Surgery, EACTS Annual Meeting, September 13 – 17, 2008, Lisbon, Portugal.

15:

3rd Fraunhofer Life Science Symposium: “Ischemia and Regeneration”. October 24 – 25, 2008. Leipzig, Germany.

16:

Gäbel R, Klopsch C, **Furlani D**, Li W, Yerebakan C, Wang W, Pittermann E, Ren-Ke Li, Nan Ma, Gustav Steinhoff, Intramyocardial Administration of Erythropoietin Promotes Cell Proliferation, Induces Early Angiogenesis and Attenuates Cardiac Remodeling. *Circulation* 2008; 118:S_875 Annual meeting of the American Heart Association, Scientific Sessions, November 8 – 12 2008 New Orleans, USA.

17:

Klopsch C, **Furlani D** , Gäbel R, Li W, Ugurlucan M, Ong LL, Ma N, Steinhoff G. Intramyocardial injection of Erythropoietin augments stem cell recruitment and restores cardiac functions in a rat myocardial infarction model. 19th Annual Meeting of the Scandinavian Society for Research in Cardio Thoracic Surgery, February 5 – 7, 2009. Geilo, Norway.

18:

Furlani D , Li W, Wang L, Donndorf P, Jungebluth P, Klopsch C, Delyagina E, Westien I, Lützow K, Lendlein A, Li RK, Steinhoff G, Ma N. Abnormal rat mesenchymal stem cells do not improve the cardiac function of the infarcted heart. 7th Annual Meeting of the International Society for Stem Cell Research, ISSCR, July 8 – 11, 2009. Barcelona, Spain.

19:

Gäbel R, **Furlani D**, Frank J, Wang L, Li W, Li RK, Steinhoff G, Ma N. Human bone marrow MSCs exhibit better cardiac regenerative capacity than cord blood MSCs. 7th Annual Meeting of the International Society for Stem Cell Research, ISSCR, July 8 – 11, 2009. Barcelona, Spain.

20:

Pittermann E, **Furlani D** , Klopsch C, Gäbel R, Li W, Wang W, Wagner K, Steinhoff G, Ma N. Erythropoietin-α patterns of stem cell chemoattractant genes regulation in cardiac myocytes and endothelial cells. "8th International Luebeck Conference on the Pathophysiology and Pharmacology of Erythropoietin and other Haematopoietic Growth Factors", July 30 – August 1, 2009. Luebeck, Germany.

21:

Furlani D, Li W, Wang L, Li R, Lendlein A, Steinhoff G, Ma N. Early passage mesenchymal stem cells could display abnormalous genotype, karyotype and metabolism. Congress of European Society of Cardiology, August 29 – September2, 2009. Barcelona, Spain.

13. Acknowledgement

This work is the results of reliable collaboration between various research institutes and scientists: Department of Cardiac Surgery, University of Rostock, Rostock, Germany (Prof. Dr. Gustav Steinhoff), Institute for Experimental Surgery, University of Rostock, Rostock, Germany (Prof. Dr. Brigitte Vollmar), Department of Pediatric Cardiology and Intensive Care, Children's Hospital, University of Rostock, Rostock, Germany (Prof. Dr. Matthias Peuster, Dr. Andreas Drynda), Institute for Cell Biology and Biosystems Technology, Department of Biology, University of Rostock, Rostock, Germany (Prof. Dr. Dieter Weiss, Dr. Sergey Kuznetsov), Institute for Transfusion medicine and Immunology, University of Heidelberg, Heidelberg, Germany (Dr. Karen Bieback), Division of Hematology and Oncology, University of Rostock, Germany (Dr. Agnes Knopp), Bionas GmbH, Rostock, Germany, Institute of Pathology, University of Rostock, Rostock, Germany, (Prof. Dr. Horst Nizze, Dr. Ulf Titze), Institute of Clinical Chemistry and Laboratory Medicine, University of Rostock, Rostock, Germany, (Dr. Christiana Zingler), Institute of Medical Informatics and Biometry, University of Rostock, Rostock, Germany, (Prof. Dr. Guenther Kundt), Zentrum für Biomaterialentwicklung, GKSS, Teltow, Germany; (Prof. Dr. Andreas Lendlein, Dr. Karola Lützow), Department of Anesthesia, Klinikum Südstadt, Rostock, Germany (Dr. Klaus Wagner), Institut für Technische und Makromolekulare ChemieLehrstuhl für Textilchemie und Makromolekulare Chemie, Aachen, Germany (Prof. Dr. Doris Klee, Robert Lösel), Division of Cardiovascular Surgery, Toronto General Hospital and the University of Toronto, Canada (Prof. Dr. Ren-Ke Li), Departments of General Thoracic Surgery, Hospital Clinic of Barcelona, University of Barcelona, Barcelona, Spain (Prof. Dr. Paolo Macchiarini, Dr. Philipp Jungebluth), I gratefully acknowledge the support of these institutions.

This work was carried out in the Laboratories for cardiac tissue and organ replacement, FKGO, Department of Cardiac Surgery, University of Rostock, Rostock, Germany. I thank all past and present colleagues for the good working atmosphere and the scientific discussions. I would like to thank Prof. Dr. Gustav Steinhoff, for supervising this thesis and providing timely assistance with the programming and proofreading. I would also like to thank Prof. Dr. Dieter Weiss for co-supervising this work. My great appreciation and thanks to my supervisor Dr. Nan Ma for suggesting the

topic, for her constant scientific and extra-scientific support and for the trust demonstrated in these years.

Thanks to Prof. Dr. Ludwig Jonas for providing me the initial and fruitful contact with Prof. Dr. Gustav Steinhoff. I am grateful to Dr. Wenzhong Li, Dr. Lee Lee Ong, Dr. Alexander Kaminski, Prof. Dr. Andreas Liebold, Dr. Peter Donndorf, Dr. Sergei Kuznetsov, Dr. Yury Ladilov, Dr. Sanjeev Kumar, Dr. Murat Ugurlucan and Dr. Philipp Jungebluth, for their friendly assistance and profitable discussion. Thanks to my colleagues and Ph.D. candidates Christian Klopsch, Weiwei Wang and Ralf Gäbel for helping me during the last years. Appreciation and encouragement to the new Ph.D. candidates of FKGO: Evgenya Delyagina, Yue Zhang, and Peter Mark. I am very grateful to all the students from the faculties of Medicine and Medical Biotechnologies which worked with me especially Erik Pittermann, Ingeborg Westien and Liang Wang. Many thanks to our great technicians: Ms. Margit Fritsche, Ms Bianca Pölchow and Miriam Nickel.

A special thank to my second family (the German one) Claudia, Stefan, Dagmar and Florian Havenstein and the Kehlenbeck that continued to sustain me during the period of my Ph.D. with warm hospitality. I would like to thank my mother Lucia Stragliati and my Italian family for the many years of education and love. Finally, I want to dedicate this work to the person that has been for me the principal reason, the main supply of energies, love and good feelings of the last seven years, Carolin Havenstein. Certainly without her this thesis would have never been written. Thank you.

14. Manuscript reprints

Available at: http://www.ncbi.nlm.nih.gov/pubmed/19449462

Manuscript 1

Intracardiac Injection of Erythropoietin Induces Stem Cell Recruitment and Improves Cardiac Functions in a Rat Myocardial Infarction Model

Klopsch C*, **Furlani D***, Gäbel R, Wagner K, Li W, Ugurlucan M, Kundt G, Zingler C, Titze U, Wang W, Ong LL, Li R, Ma N, Steinhoff G

J Cell Mol Med. 2009 Apr;13(4):664-79

*Equal contribution

2009

Available at: http://www.ncbi.nlm.nih.gov/pubmed/18555090

Manuscript 2

Intracardiac erythropoietin injection reveals antiinflammatory potential and improved cardiac functions detected by Forced Swim Test

Furlani D, Klopsch C, Gäbel R, Ugurlucan M, Pittermann E, Klee D, Wagner K, Li W, Wang W, Ong LL, Nizze H, Titze U, Lutzow K, Lendlein A, Steinhoff G, Ma N

Transplant Proc. 2008;40:962-6

2008

Available at: http://www.ncbi.nlm.nih.gov/pubmed/19249320

Manuscript 3

Is the intravascular administration of mesenchymal stem cells safe?

Furlani D, Ugurlucan M, Ong LL, Bieback K, Pittermann E, Westien I, Wang W, Yerebakan C, Li W, Gäbel R, Li R-K, Vollmar B, Steinhoff G, Ma N

Microvascular Research. 2009 May;77(3):370-6

2009

Available at: http://www.ncbi.nlm.nih.gov/pubmed/19558780

Manuscript 4

A transformed cell population derived from cultured mesenchymal stem cells has no functional effect after transplantation into the injured heart

Furlani D, Li W, Pittermann E, Klopsch C, Wang L, Knopp A, Jungebluth P, Thedinga E, Havenstein C, Westien I, Ugurlucan M, Li R, Ma N, Steinhoff G

Cell Transplantation. 2009;18(3):319-31

2009

University of Rostock